# EXPRESSION OF PROSTAGLANDIN ENDOPEROXIDE SYNTHASE IN THE FETAL BRAIN AND INTERACTIONS OF PROSTANOIDS AND STRESS HORMONES DURING CEREBRAL HYPOPERFUSION

By

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*This dissertation is dedicated to my parents and brother at home, who encouraged me to reach high . . .*

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# EXPRESSION OF PROSTAGLANDIN ENDOPEROXIDE SYNTHASE IN THE FETAL BRAIN AND INTERACTIONS OF PROSTANOIDS AND STRESS HORMONES DURING CEREBRAL HYPOPERFUSION

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Fetal sheep respond to hypotension with reflex hormonal and hemodynamic responses which return blood pressure to normal levels. Some of these responses are mediated by both baroreceptor- and chemoreceptor- reflexes. However, a significant portion of the reflex responses are not mediated by either baroreceptors or chemoreceptors. We hypothesized that cerebral hypoperfusion reduces fetal cerebral blood flow (CBF), and that reductions in CBF stimulate prostanoids biosynthesis within the central nervous system which, in turn, stimulate adrenocorticotropin (ACTH), arginine vasopressin (AVP) and cardiovascular responses.

Fetal sheep were chronically catheterized, either intact or subjected to a prior carotid sinus denervation between 124 and 136 days gestation. The cyclooxygenase

inhibitor indomethacin (0.2 mg/kg) or its vehicle was injected intravenously 90 min before the start of a 10-min period of hypotension. Hypotension induced by vena caval occlusion significantly increased ACTH and AVP secretion, and indomethacin significantly reduced the magnitude of this increase. Baroreceptor and chemoreceptor denervation attenuated the ACTH and AVP responses, but these responses were not further inhibited by indomethacin. This suggested that endogenous prostanoids partially mediate the reflex hormonal responses to hypotension. Prostaglandin $E_2$ and thromboxane $A_2$ were produced in the brain in response to cerebral hypoperfusion induced by brachiocephalic occlusion. Cerebral hypoperfusion significantly reduced CBF and indomethacin decreased the reduction of CBF by inhibiting local prostanoid production. The increase in prostanoid production was associated with the "suicide" inactivation of prostaglandin endoperoxide synthase (PGHS), which is the key enzyme for prostanoid biosynthesis. Western blot and RT-PCR analyses showed that in the brain protein levels and mRNA of PGHS-2, but not PGHS-1, were induced by cerebral hypoperfusion. Immunocytochemistry demonstrated that immunoreactive PGHS-2 and Fos induced by cerebral hypoperfusion, were localized in neurons and the cerebrovasculature.

In summary, these studies indicated that hypotension stimulated ACTH and AVP secretion, also induced expression of mRNAand protein for prostaglandin endoperoxide synthase-2 in the fetal brain. We conclude that the induction of PGHS-2 is the major contributor to elevated prostanoids in the brain which, in turn, modulate the ACTH and AVP responses to cerebral hypoperfusion in late-gestation fetal sheep.

# CHAPTER 1
# INTRODUCTION

Hypotension initiates hormonal and hemodynamic responses which act in concert to return blood pressure to normal levels. Many of these responses are mediated by cardiovascular mechanoreceptors which change their firing rates in response to changes in vessel wall or cardiac chamber radius. Cardiovascular mechanoreceptors, situated in the walls of arteries, in the walls of cardiac atria and ventricles, and elsewhere, respond to stretch with increased rates of firing. Arterial baroreceptors and chemorecepotors play an important role in maintaining blood pressure during periods of hypotension in late gestation fetal sheep (Wood, 1989; Wood et al. 1990). However, baroreceptors and chemoreceptors do not completely mediate the responses to hypotension in the fetus. AVP, ACTH, and renin reflex responses to hypotension (produced by vena caval obstruction) were only 50% inhibited by peripheral baroreceptor and chemoreceptor denervation (Wood, 1989). Therefore, this observation suggests that some other mechanisms within the central nervous system are responsible for the remainder of the reflex endocrine responses to hypotension in the fetus.

Moderate to severe hypotension in the fetus reduces cerebral blood flow and reductions in fetal cerebral blood flow are effective in increasing cardiac output and regional vasoconstriction (Backofen et al. 1991). Therefore, it is likely that some of the

reflex responses to moderate to severe hypotension in the fetus are the result of compromised fetal cerebral perfusion. Koos et al. (1994) demonstrated that fetal hypoxia stimulates the production of adenosine, and that some of that adenosine could originate from the cerebral vasculature and, as such, could represent a transduction mechanism for stimulating AVP responses to hypoxia. Prostanoids are another class of compounds which could be stimulating hormonal reflex responses to hypotension in the fetus. The influence of prostanoids on the central nervous system to alter hormonal and hemodynamic responses might mediate the homeostatic responses to cerebral ischemia. At present, the mechanisms of these responses to reductions in cerebral blood flow are not fully understood. Prostanoids appear to contribute to regulation of the cerebral vascular tone in human beings and some animals, particularly during the perinatal period (Pourcyrous et al. 1994). One site of prostanoid production is in the local cerebrovasculature during cerebral hypoperfusion (Chemtob et al. 1990a). During hypotension, brain and sagittal sinus concentrations of vasodilator prostaglandins have been show to increase to maintain cerebral blood flow (Leffler and Busija, 1987a; Leffler et al. 1986; Chemtob et al. 1990b). But concentrations of the vasoconstrictor thromboxane are also increased in newborn piglets, suggesting the participation of thromboxane in the decrease in cerebral blood flow during hypotension (Chemtob et al. 1990b). However, in this research project, we proposed that in addition to their direct action at the cerebral vasculature, these prostanoids can be generated within the fetal brain in responses to cerebral hypoperfusion, and that these prostanoids in turn modulate the reflex cardiovascular and endocrine responses which increase blood pressure and ultimately help ameliorate the cerebral hypoperfusion.

## Hypothesis

Studies from Wood's laboratory and others demonstrate that: 1) prostaglandin $E_2$ and thromboxane $A_2$ act at the central nervous system to increase ACTH and AVP secretions; 2) reductions in cerebral blood flow cause the local production of these prostanoids in the brain; 3) baroreceptors and chemoreceptors do not completely mediate the hormonal reflex responses to hypotension. Other mechanisms responsible for this reflex response are not fully known yet. In this project, we hypothesized that changes in fetal cerebral perfusion pressure stimulate increases in ACTH and AVP secretions and cardiovascular responses, by the induction of prostaglandin endoperoxide synthase, resulting in increased local generation of prostaglandin $E_2$ and thromboxane $A_2$.

## Specific Aims

The overall objective of this research project is to explore the mechanism that cerebral hypoperfusion stimulates the production of prostaglandin $E_2$ and thromboxane $A_2$ within the brain and that these prostanoids in turn mediate the reflex endocrine and cardiovascular responses to this stimulus in the late-gestation fetal sheep. My specific objectives are as follows:

1) determine if endogenous prostaglandin $E_2$ and thromboxane $A_2$ modulate the ACTH and AVP responses to hypotension

2) characterize the effects of endogenous prostaglandin $E_2$, thromboxane $A_2$ and indomethacin on the reduction of cerebral blood flow

3) elucidate the mechanism responsible for the prostanoids biosynthesis during cerebral hypoperfusion

a) test the inducibility of prostaglandin endoperoxide synthase-2 mRNA and protein by cerebral hypoperfusion.

b) localize and evaluate the immunoreactive prostaglandin endoperoxide synthase and Fos in the brain in response to cerebral hypoperfusion.

# CHAPTER 2
# LITERATURE REVIEW

## Baroreceptors and Chemoreceptors Control of Hormone Secretion

Baroreceptors and chemoreceptors reflex systems are important mechanisms involved in the rapid control of blood pressure in both adult and fetal animals. It is known that arterial baroreceptors in the carotid sinus and aortic arch buffer the minute-to-minute changes in arterial blood pressure which are produced by common disturbances in blood pressure, including orthostasis and psychogenic stress (Cowley et al. 1973). A change in pressure signals are transmitted from each carotid sinus through the Hering's nerve to the glossophryngeal nerve and hence to the tractus solitarius (NTS) in the medullary area of the brain stem. Signals from the arch of the aorta are transmitted through the vagus nerves also into NTS of the medulla (Guyton, 1991). Carotid baroreceptor cell bodies are located in the inferior ganglion of the glossopharyngeal nerve, and aortic baroreceptor cell bodies are located in the inferior ganglion of the vagus nerve. Neurons in the NTS subseving cardiovascular system regulation occupy primarily the densely packed portion of the nucleus near the obex (Eckberg and Sleight, 1992). Afferent baroreceptor neurons carrying the incoming signals release chemicals that alter properties of other neurons and eventually provoke changes that affect parasympathetic and sympathetic outflow. After the baroreceptor signals have entered the NTS of the medulla, secondary signals modulate

the vasoconstrictor center and the vagal center in the medulla in response to the incoming signals. Anatomical studies identified monosynatic interconnections within the NTS, as well as between NTS and the hypothalamus (Spyer, 1972), the rostral ventrolateral medulla (Sawchenko and Swanson, 1982) and the intermediolateral region of the spinal cord (Loewy and Burton, 1978). Feedback signals are then sent back through the efferent autonomic nervous system to the peripheral tissues such as arterioles, veins and heart to adjust arterial blood pressure toward the normal level on a second to second basis. Prostaglandin endoperoxide synthase are found in various regions that are described above, especially in the hypothalamus and in the NTS (Breder et al. 1992, 1995).

It is also well accepted that both baroreceptors and chemoreceptors influence the secretion of the hormonal systems which are involved in blood pressure regulation. For example, arterial baroreceptors tonically inhibit the secretion of vasopressin (Share, 1965), ACTH and cortisol (Wood and Rudolph, 1983; Wood et al. 1984), and renin and aldosterone (Bartter et al. 1960; Thames et al. 1978). Peripheral chemoreceptors are thought to control the secretion of AVP (Hanley et al. 1988) and ACTH (Raff et al. 1984). Increases in plasma Ang II and vasopressin promote vasoconstriction and direct defense of blood pressure (Iwamoto and Rudolph, 1981; Schwartz and Reid, 1981; Robillard et al. 1982). Increases in plasma Ang II, aldosterone and vasopressin promote renal sodium and water conservation (Bie, 1990; Hall and Brands, 1992) and an increase in plasma cortisol concentration promotes restitution of plasma proteins (Pirkle and Gann, 1975) and intracellular-extracellular compartment fluid shifts (Bartter and Fourman, 1962; Fan et al. 1978).

In late-gestation fetus, decreases in arterial blood pressure increase the secretion of ACTH, cortisol, AVP, and renin (Robillard et al. 1979; Rose et al. 1981; Wood, 1989). The magnitudes of the responses are proportional to the magnitude of the decrease in arterial pressure (Wood et al. 1982), and the responses are attenuated by prior sinoaortic denervation (Wood, 1989). AVP secretion was not affected by arterial chemoreceptor activity in the fetus (Raff et al. 1991). Hypercapnia in combination with hypoxia stimulated increases in fetal plasma renin activity which were blocked by sinoaortic denervation (Wood et al. 1990). Reductions in blood flow to the arterial chemoreceptors could stimulate reflex ACTH responses to acute hypotension in fetuses (Wood, 1995a). Because chemoreceptors can not account for the responses to hypotension, and sinoaortic denervation attenuates the responses of the three endocrine systems (ACTH, AVP, and renin), it was concluded that at least some of the endocrine responses to acute hypotension must be mediated by arterial baroreceptors (Itskovitz and Rudolph, 1982; Wood, 1995a). Wood (1995a) suggested that the baroreceptors are indeed active in late gestation, and that unloading of the baroreceptors stimulates reflex responses. In intact fetal sheep arterial baroreceptors buffer changes in arterial blood pressure caused by orthostasis, cord compression, and other acute influences, and sinoaortic denervation eliminates the buffer (Wood, 1995a).

Based on this information, it is concluded that both baroreceptors and chemoreceptors located in the carotid sinus and/or aortic arch contribute to the overall reflex responses to acute hypotension. A portion of the AVP response to acute hypotension is mediated by arterial baroreceptors and not by chemoreceptors, ACTH

responses might also be mediated by arterial baroreceptors. However, AVP and ACTH reflex responses to hypotension (produced by vena caval obstruction) were only 50% inhibited by peripheral baroreceptor and chemoreceptor denervation (Wood, 1989). Therefore, some other mechanism contributed to these reflex responses that need to be investigated.

## Prostanoid Control of Hormone Secretion

Prostanoids are a class of compounds which could be stimulating hormonal reflex responses to hypotension in the fetus. One site of production of prostanoids is in the local cerebrovasculature during cerebral hypoperfusion (Chemtob et al. 1990a), but in addition to being made by and released into the vasculature, prostanoids are synthesized within neurons in the brain of fetal sheep (Jones et al. 1993; Pace-Asciak and Rangaraj, 1976). Physiologically, this endogenous production of prostanoids can be demonstrated by central administration of indomethacin to late-gestation fetal sheep at doses which block CNS prostaglandin synthesis, but not peripheral prostaglandin synthesis. Prostanoids might alter cardiovascular function by affecting neuronal processing within the central nervous system. For example, Breuhaus and coworkers (Breahaus and Chimosky, 1983; Breahaus and Chimosky, 1985; Breahaus et al. 1989) and Cudd and Wood (1991) have demonstrated that infusions of $PGE_2$ into the carotid arteries of conscious adult sheep increase heart rate and blood pressure. This effect is not mediated by an action of $PGE_2$ on arterial baroreceptor or chemoreceptor afferent activity (Breahaus and Chimosky, 1983) and is therefore a direct effect on the brain. $PGE_2$ also has a direct effect on the

fetal brain to alter blood pressure and heart rate, presumably by altering autonomic efferent tone (Cudd and Wood, 1991; Cudd and Wood, 1992). Wood and coworkers (1993) have found that in addition to its action in the peripheral circulation, thromboxane also stimulates increases in arterial blood pressure and heart rate by an action in regions perfused by carotid artery.

An important component of the effect of prostanoids on the cardiovascular system is their effect on the hormones which, in turn, influence blood pressure or fluid balance. It has been demonstrated that $PGE_2$ has direct effect on the fetal sheep pituitary gland by enhancing AVP-stimulated, but not CRH-stimulated ACTH secretion from dispersed fetal anterior pituitary cells in culture (Brooks and Gibson, 1992). Young and Thorburn (1994) have found that $PGE_2$ has potent stimulatory actions on the gestation fetal sheep pituitary to increase both the absolute concentration and the bioactive fraction of ACTH-containing peptides in the fetal circulation. An increase in the concentration of $PGE_2$ in the hypothalamus, therefore, would be expected to stimulate ACTH secretion (Wood, 1995a). It also directly stimulates glucocorticoid secretion from the fetal adrenal gland. It has also showed that $PGE_2$ increases AVP secretion (Inoue et al. 1991). Intracerebroventricular infusion of $PGE_2$ can stimulate ACTH and cortisol secretion in fetal sheep (Brooks, 1992) and treatment with indomethacin decreases ACTH release (Thompson and Hege, 1978). Thromboxane $A_2$ is also a potential mediator of CRF release. In vitro treatment of adult rat hypothalami with U46619 (a thromboxane $A_2$ mimic) causes secretion of corticotropin-releasing factor (CRF) (Bernardini et al. 1989). Wood et al. have found that thromboxane $A_2$ is a powerful and specific stimulator of ACTH secretion in both adult and fetal sheep

(Cudd and Wood, 1993; Cudd and Wood, 1994; Wood et al. 1993). In the adult sheep, endogenously generated thromboxane $A_2$ stimulates ACTH secretion, and in the fetal sheep infusions of U46619 into the carotid arterial blood supplying the fetal brain stimulates increases in fetal ACTH secretion (Wood et al. 1993). The ACTH response to this stimulus is blocked through the inhibition of cyclooxygenase or through blockade of $TxA_2$-PGH receptor (Cudd and Wood, 1993; Cudd and Wood, 1994). Whether endogenous prostanoids could mediate the ACTH and AVP secretions in response to hypotension in the fetus remains to be found.

## The Relationship Between Prostanoids and Cerebral Blood Flow

In fetal and newborn animals, cerebral blood flow (CBF) is maintained during changes in arterial blood pressure (Chemtob et al. 1990a; Hernandez et al. 1980; Papile et al. 1985; Tweed et al. 1983). In the fetal sheep, cerebral blood flow is held relatively constant during changes in arterial blood pressure between 45 and 80 mm Hg(Papile et al. 1985; Purves and James, 1969; Tweed et al. 1983). Therefore, decreases in arterial blood pressure below the normally regulated level result in a decrease cerebral blood flow.

Prostanoids appear to contribute to regulation of cerebral vascular tone in human beings and some animals, particularly during the perinatal period. Substantial physiological data *in vivo* point to a significant role for dilator prostanoids in the regulation of cerebral vascular tone and cerebral blood flow in newborns (Leffler et al. 1993). The decreases in CBF occur concomitantly with decreases in CSF dilator prostaglandin levels (Pourcyrous et al. 1990; Leffler et al. 1989). Chemtob and his

coworkers (1990b) demonstrated that reduction of cerebral blood flow in the newborn piglet below the autoregulatory range stimulates increases in the local production of $PGE_2$, $PGF_{2\alpha}$, and thromboxane $B_2$.

Arachidonic acid is liberated from damaged cell membranes during ischemia and is the source of vasoactive prostanoids which are believed to adversely influence cerebral ischemia. When cerebral blood flow is reduced, both dilator and constrictor prostanoids are produced locally. The physiological effects of arachidonate metabolites often have opposing actions. For example, $PGE_2$ and $PGI_2$ are potent vasodilators, while $TxA_2$ and $PGF_{2\alpha}$ are vasoconstrictors (Moncada and Vane, 1978; Moskowitz and Coughlin, 1981). The prostanoids are most likely a component of the control of vascular tone by locally generated effectors of tone. Chemtob et al. (1990c) have shown that $PGE_2$, $PGF_{2\alpha}$, and $PGI_2$ increase CBF in newborn piglets. This effect is in sharp contrast to that observed in adults of virtually all species studied, in which $PGE_2$ and $PGF_{2\alpha}$ are cerebral vasoconstrictors (White and Hagen, 1982; Murphy and Pearce, 1988). Thus, there exists a predominant vasodilator action of the major cerebrovascular PGs in newborns. This may compromise CBF autoregulation as appropriate vasoconstriction is required to maintain CBF when blood pressure increases.

Inhibition of cyclooxygenase, an enzymatic step in arachidonic acid metabolism, has an improvement effect on the outcome during cerebral ischemia. Indomethacin, a cyclooxygenase inhibitor, has been used for pharmacologic closure of the patent ductus arteriosus (Heymann et al. 1976; Firedman et al. 1976; Gersony et al. 1983), and it has a potential ability to prevent or attenuate the development of intraventricular hemorrhage

(Ment et al. 1994). Free indomethacin crosses the blood brain barrier (Bannwarth et al. 1990). It can be found in the brain within 30 min of systemic administration (Bannwarth et al. 1990; Day et al. 1987). It is generally assumed that the duration of action of indomethacin is long, but the plasma half-life is relatively short and the CSF concentration is closely related to that of plasma (Bannwarth et al. 1990). Indomethacin has been reported to have beneficial prophylactic effects in prevention and/or reduction of severity of intraventricular /periventricular hemorrhage in premature babies (Ment et al. 1985a; Hanigan et al. 1988; Bada et al. 1989). In piglets, indomethacin decreases CBF at rest (Leffler et al. 1985). These decreases in CBF occur concomitantly with decreases in CSF levels of dilator PGs (Leffler et al. 1989).

The effects of prostanoids and indomethacin on cerebral ischemia on fetuses are not known. We hypothesized that during cerebral hypoperfusion, prostanoids produced in the microvasculature and neurons might cause vasoconstriction and reduce cerebral blood flow, and that pretreatment with indomethacin may attenuate this reduction of cerebral blood flow. The information obtained from this study may result in better understanding and treatment of fetal cerebral ischemia.

## Prostanoids Biosynthesis

When a cell-specific stimulus (hormone or growth factor) interacts with the appropriate receptors, the subsequent signal transduction results in activation of phospholipases. Two pathways are postulated to be responsible for the release of unesterfied arachidonate from cell membrane. One involves the direct hydrolysis of

arachidonic acid from phospholipids by the action of phospholipase $A_2$ (Rittenhouse-Simmons et al. 1976; McKean et al. 1981; Isakson et al. 1977). The other begins with the action of phosphatidylinositol-specific phospholipase C, followed by the action of diglyceride lipase and monoglyceride lipase to liberate arachidonic acid (Chau and Tai, 1981; Borgeat and Samuelsson, 1979).

Once released, arachidonic acid is metabolized through three different pathways. The first pathway involves a series of lipoxygenases (5, 12 or 15-lipoxygenase) that peroxidize arachidonic acid at different carbon atoms to form unstable hydroperoxyeicosatetraenoic acids (HPETEs) which are then converted into lipoxins (LXs), hydroxyeicodatetraenoic acids (HETEs), leukotrienes (LTs) and related compounds (Borgeat and Samuelsson, 1979; Fitzpatrick and Murphy, 1988). The second pathway is known as "epoxygenase branch" of the arachidonate cascade and is mediated by cytochrome P450 monooxygenases (Shimizu and Wolfe, 1990). The third pathway involves prostaglandin endoperoxide synthase (PGHS or cyclooxygenase) that leads to the formation of endoperoxide $PGH_2$. $PGH_2$ is the common intermediate for the biosynthesis of PGs, prostacyclin ($PGI_2$) and thromboxane $A_2$ ($TxA_2$) (DeWitt et al. 1981; Hamberg et al. 1975). After $PGH_2$ is biosynthesized, the various eicosanoids are made in a tissue-specific fashion before they exit the cell (Fig. 2-1). Each eicosanoid can bind to multiple receptors on either the cell of origin or neighboring cells. The eicosanoid-binding receptors have not been fully characterized, but so far the evidence indicates that they are all G-protein linked (Smith, 1989). Each cell type has various receptors with distinct functions. Each receptor signals a different G-protein to alter the production of second

messengers (Smith and Marnett, 1991; Smith, 1989). The prostaglandins thereby help to coordinate the cellular response to circulating hormones (Smith and Marnett, 1991; Smith, 1989).

The products of epoxygenase, lipoxygenase and cyclooxygenase pathways have a wide range of biological activities. Eicosanoid is used to refer to this large family of compounds. Several epoxygenase metabolites have been identified as endogenous constituents of mammalian systems. Some of them were found to be potent inhibitors of $Na^+$ $K^+$-ATPase in the corneal epithelium or to induce glucagon or vasopressin secretion (Shimizu and Wolfe, 1990; Whittake et al. 1976). Thus, the epoxygenase metabolites of arachidonic acid might be involved in the regulation of renal and ocular transport functions which rely on the sodium pump mechanism. Prostaglandins can influence arterial and venous blood flow, capillary permeability, tone of smooth muscle, cell chemotaxis and sensation of pain. Leukotrienes participate in host defense reactions and pathophysiological conditions such as immediate hypersensitivity and inflammation.

The biological activities of the eicosanoids are mainly limited to their local site of biosynthesis. Thus, they are so called local hormones or autocoids. These eicosanoids usually do not exert systemic effects because they are rapidly metabolized to inactive metabolites. Some eicosanoids such as $TxA_2$ and $PGI_2$ are chemically unstable, with half life measured in seconds, and undergo rapid non-enzymatic hydrolysis to inactive compounds $TxB_2$ and 6-keto-$PGF_{1\alpha}$ (Hamberg et al. 1975). Others are enzymatically degraded to inactive compounds. The enzymatic degradation of eicosanoids occurs in multiple organs of the body including the liver, lung, kidney, blood vessels, platelets and erythrocytes. Liver and lung are the most important sites of metabolism.

A contemporary view of eicosanoid regulation would consider two additional processes. The first is transcriptional and translational control of enzyme expression. The second is the mechanism-based inactivation of eicosanoid biosynthetic enzymes. Increasing evidence supports the role of gene regulation and enzyme inactivation as important control mechanisms in eicosanoid metabolism.

Prostaglandin Endoperoxide Synthase

Prostaglandin endoperoxide synthase (PGHS) converts arachidonic acid to $PGH_2$, the precursor of prostaglandins, thromboxanes, and prostacyclin. This enzyme is present in all mammalian cells that utilize these classes of eicosanoids (Smith and Marnett, 1991). Small amounts of eicosanoids are produced locally and have effects only on those cells in the immediate vicinity which express specific prostaglandin receptors. Eicosanoids have been shown to have effects on almost every physiological system, including the reproductive, digestive, cardiovascular, immune, nervous and respiratory systems. In these systems, cells that make eicosanoids typically synthesize one or two specific products rather than participate in the entire arachidonic acid cascade. For example, platelets primarily produce $TxA_2$, and endothelial cells primarily produce prostacyclin ($PGI_2$). Such bioactive lipids have both autocrine and paracrine functions.

PGHS can be induced in cultured cells by growth factors (EGF), hormones, and tumor promoters with increases in prostaglandin production (Yokota et al. 1986; Kusaka et al. 1988; Raz et al. 1989; Pash and Bailey, 1988; Wu et al. 1988; Goerig et al. 1989). This increased activity is not attributed to increased arachidonate availability and is diminished by cycloheximide and actinomycin D, indicating that protein synthesis is

necessary for the effect. The mechanism for inducing capability has been attributed to increases in PGHS message or enzyme mass following stimulation.

Properties of PGHS

PGHS was first purified from microsomes of ovine and bovine vesicular glands (Miyamoto et al. 1976; Van der Ouderaa et al. 1977; Hemler et al. 1976). Purified PGHS exhibits a cyclooxygenase activity that oxygenates arachidonic acid to produce the hydroperoxy endoperoxide $PGG_2$ and a peroxidase activity that reduces the hydroperoxide to the corresponding alcohol, $PGH_2$ (Miyamoto et al. 1976). Purified protein has a molecular weight of 70 kDa when analyzed on denaturing electrophoresis gels (Hemler et al. 1976). However, when purified synthase is applied to a gel filtration column, it elutes as a homodimer at an apparent molecular weight of 140 kDa. Gel filtration of PGHS can be carried out in buffer containing 6 N guanidine.HCl to interrupt non-covalent interactions, but the purified protein still elutes as a homodimer under these conditions (Ogino et al. 1978).

The DNA sequence for PGHS has been elucidated from sheep, mice, and human sources (DeWitt and Smith, 1988; Merlie et al. 1988; Yokoyama et al. 1988; Yokoyama and Tanabe, 1989; DeWitt et al. 1990). Analysis of the protein sequence reveals approximately 90% sequence similarity between these species. Recent evidence suggests that there are two different RNA transcripts for PGHS, one of which is mitogen-inducible (Xie et al. 1991; Rosen et al. 1989). These two transcripts appear to be derived from different genes (Rosen et al. 1989). The mitogen-inducible form in chicken embryo fibroblasts shares 59% sequence similarity with the sheep enzyme (Xie et al. 1991).

PGHS is a membrane protein that is associated with the endoplasmic reticulum and nuclear membrane in Swiss mouse 3T3 fibroblasts (Miyamoto et al. 1976; Van der Ouderaa et al. 1977; Hemler et al. 1976; Rollins and Smith, 1980). The cyclooxygenase active site is reported to be on the cytosolic side of the endoplasmic reticulum (DeWitt et al. 1981). Detergents are required to release PGHS from the microsomal membrane, which has been interpreted to indicate that it is an integral membrane protein. However, the possibility that it is a peripheral membrane protein can not be ruled out. Five hydrophobic regions are deduced from a hydrophobicity profile of the primary amino acid sequence. One of these regions is in the signal peptide (Yokoyama et al. 1988). There is no obvious transmembrane segment in PGHS because none of the hydrophobic regions have average hydrophobicities characteristic of a transmembrane sequence nor are they long enough to span the membrane (DeWitt and Smith, 1988; Merlie et al. 1988; Yokoyama et al. 1988).

PGHS is a glycoprotein. Approximately 5 mol of N-acetyl glucosamine and 12 mol of mannose are contained on each 70 kDa subunit (Van der Ouderaa et al. 1977). The attachment of these carbohydrates accounts for the discrepancy between the 65.5 kDa molecular weight determined from the primary amino acid sequence and the molecular size of 70 kDa determined from its electrophoretic mobility (DeWitt and Smith, 1988; Merlie et al. 1988; Yokoyama et al. 1988). Four potential N-glycosylation sites exits in the amino acid sequence (Merlie et al. 1988; Yokoyama and Tanabe, 1989). There is firm evidence that two of these sites are glycosylated in the purified enzyme. Both are in the N-terminal half of the protein (Smith and Marnett, 1991).

Cyclooxygenase and Peroxidase Activities of PGHS

Cyclooxygenase catalyzes the oxygenation of arachidonate to the prostaglandin endoperoxide $PGG_2$ (Smith and Marnett, 1991; Marnett and Maddipati, 1991). The PGHS peroxidase activity catalyzes the reduction of the cyclooxygenase product $PGG_2$ to $PGH_2$ (Miyamoto et al. 1976). $PGH_2$ is further converted by specific synthases (isomerases) or reductases to the major biologically active prostanoids $PGD_2$, $PGE_2$, $PGF_{2\alpha}$, $PGI_2$ and thromboxane $A_2$ (Smith et al. 1991). The peroxidase activity and cyclooxygenase activity have distinct active sites on PGHS. Aspirin, which inhibits cyclooxygenase activity, does not inhibit peroxidase activity (Roth et al. 1975; Roth and Siok, 1978; Roth et al. 1980; Raz and Needleman, 1990).

Isoforms of PGHS

Two different isoforms of PGHS were confirmed at the protein (Habib et al. 1993) and mRNA levels (O'Neill and Ford-Hutchinson, 1993). There are two distinct genes which encode for PGHS-1 (or COX-1) and PGHS-2 (or COX-2). Inducible PGHS-2 encoded by a 4 kilobase mRNA, and constitutive PGHS-1 encoded by a 2.8 kilobase mRNA. Promoter analysis suggests that PGHS-1 is a member of the housekeeping gene family (Wang et al. 1993) and PGHS-2 is a member of immediate early gene (IEG) family (Xie et al. 1993). The promoter sequence of PGHS-2 gene contained a TATA box and a variety of enhancer elements, including an activator protein-1 (AP-1) site (Xie et al. 1993). The PGHS-2 gene may be transcriptionally regulated at its AP-1 binding element

by transcription factor c-fos (Chiu et al 1988; Xie et al. 1993); however, promoter analysis of the PGHS-1 gene reveals no putative AP-1 domain (Wang et al. 1993).

PGHS-1 is the isoenzyme which was first purified from bovine and ovine microsomes and is a homodimer consisting of two subunits each of approximately 70 kilodaltons (Samuelsson et al. 1978). The deduced amino acid sequences of PGHS-1 from sheep, human, and mouse are ~90% identical at the amino acid level, with the major differences being found in the signal peptide and the 12 amino acids immediately preceding an endoplasmic reticulum retention signal at the COOH-terminal (Smith, 1992). PGHS-1 is constitutively expressed in most tissues. It is linked to basal synthesis of prostanoids during cell differentiation and it is not suppressed by corticosteroid hormones (DeWitt, 1991). Recently, however, it has been shown that PGHS-1 can be slightly induced, contributing to the up-regulation of prostanoid biosynthesis during monocyte differentiation (Hoff et al. 1993). The other isoenzyme, PGHS-2, is an inducible, transient form of PGHS. PGHS-2 was originally isolated as a v-*src*-inducible gene product in chicken fibroblasts (Simmons et al. 1991; Xie et al. 1991) and as a phorbol ester-inducible immediate early gene product called TIS10 in murine 3T3 cells (Kujubu et al. 1991). It can be induced by a variety of stimuli such as mitogens, growth factors (Kujubu and Herschman, 1992), inflammatory agents (Lee et al. 1992), hormones (Wong and Richards, 1992) and physiological activity (Yamagata et al. 1993), suggesting its involvement in physiological responses. PGHS-2 contains an 18-amino acid insert very near the COOH-terminal of the enzyme, which is an insert that is not present in PGHS-1. The remainder of the sequence of PGHS-2 is quite similar to that of PGHS-1 (Smith, 1992). PGHS-2

mRNA is detectable in brain, prostate, and lung, but it is in very low or undetectable abundance in kidney and most other major organs (Simmons et al. 1991).

The two PGHS isoforms differ in tissue distribution. Breder et al. (1992; 1995) have demonstrated that the distribution of PGHS-2 in the central nervous system was quite different from PGHS-1. One study demonstrated that PGHS-2 is the predominant form of prostaglandin endoperoxide synthase in the brain of newborn piglet (Peri et al. 1995). PGHS-1 and PGHS-2 mRNAs are expressed equally in most human tissues except that PGHS-2 mRNA has a higher level in lung tissues (O'Neill and Ford-Hutchinson, 1993).

Prostaglandin Receptors

The diversity of the effects of prostanoids is explained by the existence of a number of distinct receptors that mediate their actions. Prostanoids exert their effects through G protein-linked receptors The receptors have been named for the natural prostaglandin for which they have the greatest apparent affinity and have been divided into five main types, designated DP ($PGD_2$), FP ($PGF_2$), IP ($PGI_2$), TP ($TxA_2$), and EP ($PGE_2$) (Campbell and Halushka, 1996). The EP receptors have been further subdivided into $EP_1$ (responsible for smooth muscle contraction), $EP_2$ (responsible for smooth muscle relaxation), $EP_3$, and $EP_4$, based on physiological and molecular cloning formation (Coleman et al. 1994; Toh et al. 1995). The prostanoid responses that have been characterized most extensively at the cellular and molecular levels are the effects of $PGE_2$ on the renal collecting duct tubule. In the cortical collecting tubule cells, $PGE_2$ has been

shown to act through pharmacologically distinct stimulatory ($EP_2$) and inhibitory ($EP_3$) PGE receptors to stimulate and inhibit cAMP formation, respectively (Nakao et al. 1989; Sonnenburg and Smith, 1988; Sonnenburg et al. 1990); $PGE_2$, presumably acting via an $EP_1$ receptor, has also been demonstrated to stimulate $Ca^{2+}$ mobilization and protein kinase C activity in the rabbit collecting tubule (Hebert et al. 1990). Recently, $PGE_2$ was found to inhibit $Na^+$-$K^+$-ATPase activity in MDCK cells (Cohen-Luria et al. 1992) via G-protein mediated receptor. The thromboxane $A_2$ effects on $Ca^{2+}$ mobilization is through the $EP_1$ receptor.

Inhibition of PGHS

Nonsteroidal anti-inflamatory drugs inhibit the cyclooxygenase activity of PGHS without inhibiting peroxidase activity (Mizuno et al. 1982). Indomethacin, fluribuprofen, and meclofenamic acid initially bind to the enzyme reversibly, competing with arachidonate for the fatty acid binding site (Kulmacz and Lands, 1985; Rome and Lands, 1975). Eventually, a stable one-to-one complex forms between these inhibitors and the 70 kDa subunit (Kulmacz and Lands, 1985; Rome and Lands, 1975). The complex of apo-PGHS and inhibitor exhibits 40-10% of the initial cyclooxygenase activity upon reconstitution with heme (Kulmacz and Lands, 1985). Before reconstitution, this complex is resistant to proteolytic digestion (Kulmacz, 1989). The complex formation which yields protease resistance forms at a dose-response similar to that required for cyclooxygenase inhibition, indicating that the complex formation resulted in both cyclooxygenase inhibition and protease resistance (Kulmacz, 1989). By preincubating PGHS with both heme and

indomethacin, the synthase becomes even more resistant to proteolysis than when it is preincubated with either compound alone. Since it is unlikely that both of these compounds directly block the cleavage area, one of them must invoke a conformational change which makes the cleavage site less accessible (Kulmacz, 1989).

Reversible cyclooxygenase inhibitors of PGHS include ibuprofen and flufenamate. These compounds also make apo-PGHS more resistant to proteases (Kulmacz, 1989). Aspirin inhibits PGHS's cyclooxygenase activity by transferring its acetyl group to $Ser^{530}$ (Roth et al. 1975). Site-directed mutagenesis demonstrates that acetylation of $Ser^{530}$ inhibits cyclooxygenase activity by placing a bulky group in a position that prevents substrate binding at the cyclooxygenase active site (DeWitt et al. 1990).

Inactivation of PGHS

In the conventional model, enzyme reacts with substrate resulting in product formation and the release of enzyme for further turnover. A "suicide" enzyme partitions substrate into an alternate process leading to inactivation and eventual depletion of enzyme (Walsh, 1984; Rando, 1984; Fersht, 1985). PGHS undergoes "suicide" inactivation during catalysis (Smith and Lands, 1972; Smith and Lands, 1971). Both cyclooxygenase and peroxidase substrates inactivate PGHS (Smith and Lands, 1972). Incubation of cyclooxygenase with arachidonic acid results in loss of cyclooxygenase activity but not peroxidase activity, concurrent with $PGH_2$ production. The chemical basis for inactivation is unknown. Both cyclooxygenase and peroxidase activities of PGHS are diminished by hydroperoxides (Hemler and Lands, 1980a). Degradation or

modification of the heme prosthetic group could account for inactivation (Hemler and Lands, 1980b; Chen et al. 1987). It is important to note that inactivation of PGHS has been demonstrated *in vivo* as well as *in vitro*, indicating that it may indeed be a physiologically relevant process (Lapetina and Cuatrecases, 1979; Kent et al. 1983).

Balancing inactivation is the gene level expression of the protein. Two general types of regulation have been studied using cultured cells as model systems. The steady-state level of PGHS in some cells is altered by agents such as PDGF and IL-1. Prostaglandin synthesis in these systems increased dramatically following stimulation by various compounds. The mechanism for this increase is presumably increased PGHS synthesis. In some instances increased prostaglandin synthesis has not been associated with increased enzyme mass.

The "suicide" inactivation appears to be a function of the cyclooxygenase reaction which only destroys the enzyme's cyclooxygenase capability. The rate of "suicide" inactivation proceeds linearly with cyclooxygenase substrate consumption and the rate differs with the fatty acid used (Smith and Lands, 1972). Therefore, self-destruction of PGHS is viewed to be intrinsic to the cyclooxygenase activity and dependent on its reaction intermediates (Hemler and Lands, 1980a).

Limited digestion of PGHS into fragments of 33 kD and 38 kD also has diminished peroxidase activity whereas the cyclooxygenase activity persists (Chen et al. 1987; Raz and Needleman, 1990). The clipped enzyme retains its molecular mass of 140 kD in solution and can still be acetylated by aspirin (Chen et al. 1987; Chen and Marnett, 1989).

Thus, regulation of prostaglandin biosynthesis is complex. The balance of genesis

and "suicide" for metabolic enzymes in the arachidonic acid cascade may play a critical role in the physiology and pathophysiology of eicosanoids.

Stimulation ( growth factors, cytokines, hormones)
↓
Increased transcription and translation of biosynthetic enzymes
↓
Increased prostaglandin formation
↓
Enzyme "suicide" inactivation
↓
Depletion of enzymatic activity.

Thromboxane Synthase

The biosynthesis of $TxA_2$ involves arachidonic acid liberation, prostaglandin endoperoxide formation through cyclooxygenase, and isomerization of $PGH_2$ under the catalysis of thromboxane synthase. In normal condition free intracellular arachidonic acid level is very low. Therefore, the rate limiting step of $TxA_2$ biosynthesis is the liberation of arachidonic acid. However, two additional processes have been identified with regulatory potential. These include the genesis of enzymes necessary for thromboxane formation and the "suicide" inactivation of thromboxane synthase during catalysis, which conforms to the criteria for mechanism-based inactivation. The enzyme heme prosthetic group appears to be affected. Enzyme purified from porcine lung and platelets is "suicide" inactivated by its physiological substrate $PGH_2$ and hydroperoxy fatty acids (Smith and Lands, 1972; Hall et al. 1986; Jones and Fitzpatrick, 1991).

$TxA_2$ plays an important role in several normal physiological processes. It is required for normal platelet aggregation which forms clots to stop bleeding. Inhibition of

$TxA_2$ formation by aspirin or blocking of $TxA_2$ action by its antagonist cause prolonged bleeding time. In addition to platelets, $TxA_2$ is found in many other tissues such as lung, spleen, brain, kidney, lymphocytes and macrophages (Hamberg, 1976; Davidson et al. 1980; Goldstein et al. 1978; Murota et al. 1978; Weidmann et al. 1978; Wolfe et al. 1976; Zenser et al. 1977). Several studies indicated that coronary circulation was affected by $TxA_2$ (Gewirtz et al. 1985; Brezinski et al. 1986; Smith et al. 1980), suggesting that $TxA_2$ might be the mediator responsible for endothelin action on blood vessels. $TxA_2$ may also participate in neurotransmission. Trachte (1986) reported that U46619, a $TxA_2$ agonist, potentiated both adrenergic force generation and norepinephrine generation in rabbit vas deferens. Further, this effect could be antagonized by SQ29548, which is a well known $TxA_2$ receptor antagonist. Smooth muscles of many tissues have been reported to be constricted in response to $TxA_2$ action. $TxA_2$ is very unstable with half life of 30 seconds in physiological solution. It is rapidly hydrolyzed into the chemically stable and biologically inactive product $TxB_2$. In addition, $TxA_2$ may also be inactivated in the lung by prostaglandin dehydrogenase to produce 15-keto-$TxA_2$ which is then quickly hydrolyzed to 15-keto-$TxB_2$. Therefore, measurement of $TxB_2$ *in vitro* provides a reliable indication of thromboxane synthase activity or *in vivo* $TxA_2$ biosynthesis. Due to the unstable nature of $TxA_2$, much of the physiological properties of this compound have been inferred from synthetic mimetics, enzyme inhibitors and receptor antagonists (Campbell and Halushka, 1996).

Thromboxane synthase was first found in platelet microsomes, later in many other tissues including lung, liver, kidney, brain, heart and placenta. The enzyme has been

purified to homogeneity from lung and platelets of different species, and found to be a monomeric 53-59 kD hemoprotein. Thromboxane synthase contain a heme prosthetic group and has been classified as a cytochrome P450 protein (Haurand and Ullrich, 1985). Western blot studies indicated that the immunoreactive enzyme varied in molecular weight from different tissues which has been shown to result from a covalent modification by its substrate.

## Summary

The above background provides a basis for studying the neuromodulatory role of prostanoids on hormonal and cardiovascular responses to cerebral hypoperfusion. We know that carotid, aortic, and cardiac afferent fibers are not the sole mediators of endocrine and cardiovascular responses to severe hypotension in the fetus. We also know that the fetus responds to reduced cerebral perfusion with reflex increases in ACTH and AVP secretions and redistribution of combined ventricular output. We believe that it is likely that prostanoids mediate at least a portion of this response. It is possible that prostaglandin $E_2$ and thromboxane $A_2$ are produced locally to adjust cerebral blood flow, and these prostanoids stimulate hormonal responses and autonomic efferent activity via paracrine/autocrine fashion. The prostanoids could be synthesized in the vasculature or in the neurons of the endocrine and cardiovascular control centers within the central nervous system. We would find that both $PGE_2$ and thromboxane $A_2$ produced in the fetal brain stimulate ACTH and AVP secretions and stimulate the cardiovascular compensations to cerebral hypoperfusion.

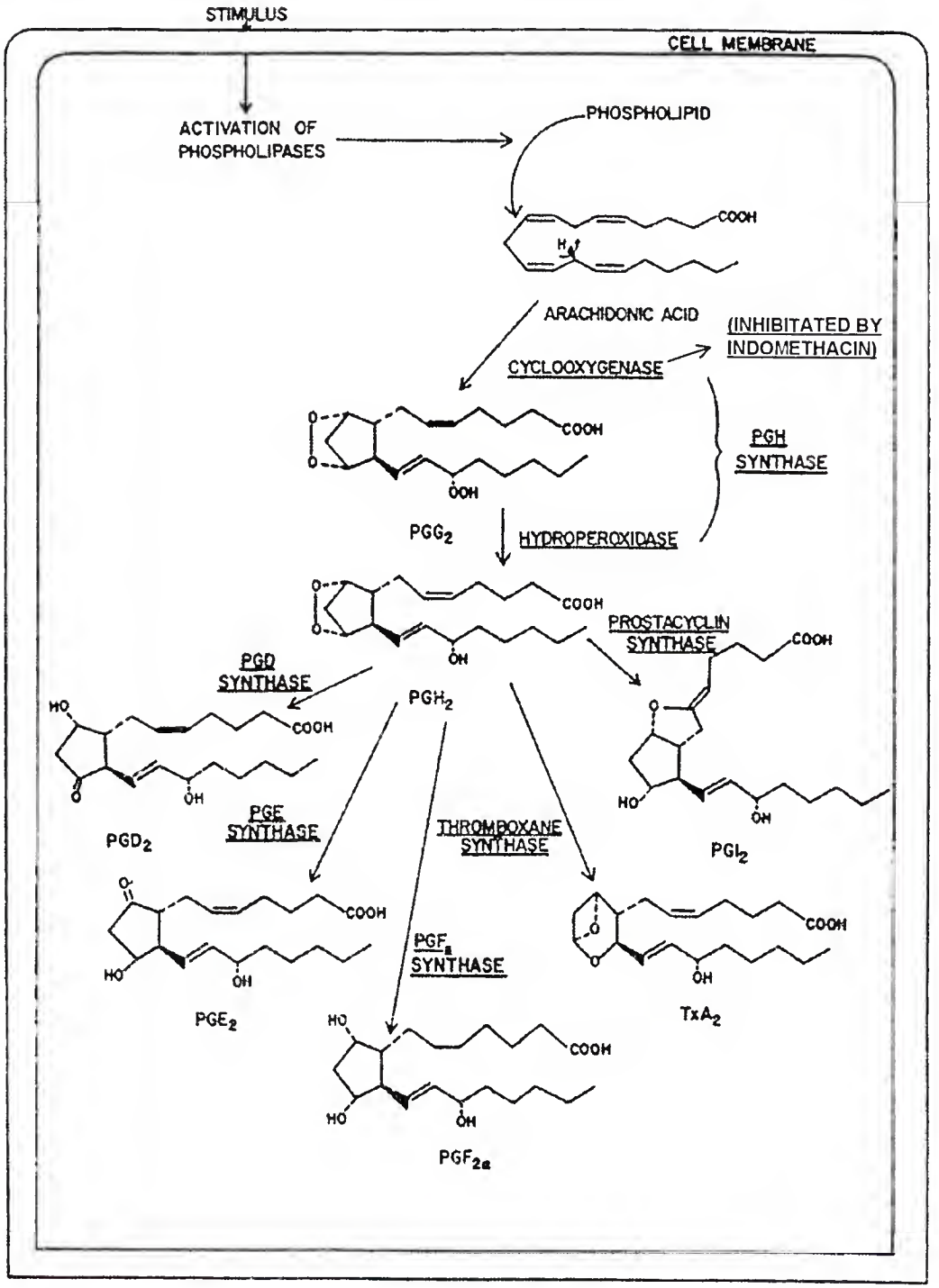



Figure 2-1. Pathways for the formation of various prostanoids. Although all products of cyclooxygenase pathway are shown, usually only one prostanoid is formed as a major product by a given cell type. $PGG_2$, $PGH_2$, $PGE_2$, $PGD_2$, $PGF_{2\alpha}$, prostaglandin $G_2$, $H_2$, $E_2$, $D_2$, $F_{2\alpha}$ respectively; $PGI_2$, prostacyclin; $TxA_2$, thromboxane $A_2$. X, indomethacin inhibits cyclooxygenase activity. Adapted from Smith (1989).

# CHAPTER 3
# ENDOGENOUS PROSTANOIDS MODULATE THE ACTH AND AVP RESPONSES TO HYPOTENSION IN LATE-GESTATION FETAL SHEEP

## Introduction

Prostanoids, including thromboxane $A_2$, prostaglandin $E_2$ and prostaglandin $I_2$, are well-known as locally generated mediators of vascular tone within the cerebral vasculature (Campbell, 1996; Heistad and Kontos, 1983, Pickard et al. 1977). We have hypothesized that, in addition to their role in the local control of cardiovascular function, prostanoids partially mediate hormonal and cardiovascular reflex responses to arterial hypotension in late-gestation fetal sheep. This hypothesis is based on the following observations: 1) arterial baroreceptors and chemoreceptors partially mediate the reflex adrenocorticotropin (ACTH) and arginine vasopressin (AVP) responses to hypotension (Wood, 1989); 2) prostaglandin $E_2$ ($PGE_2$) and thromboxane $A_2$ ($TxA_2$), when infused into the blood perfusing the brain, stimulate endocrine and cardiovascular responses reminiscent of reflex responses to hypotension (Cudd and Wood, 1992; Cudd and Wood, 1993); 3) hypotension stimulates the local synthesis and release of $PGE_2$ and $TxA_2$ in the cerebral vasculature of the newborn piglet, and therefore might have a similar effect in the late-gestation fetal sheep (Chemtob et al. 1990b).

We designed the present study to test the hypothesis that moderate hypotension, reduction of fetal arterial blood pressure approximately 50% below normally controlled levels, stimulates endocrine responses which are mediated, in part, by endogenously generated prostanoids and, in part, by chemoreceptors and baroreceptors in the carotid sinus region and in areas innervated by afferent fibers in the vagosympathetic trunks. Specifically, this study was designed to identify the effects of denervation alone and the effects of prostaglandin synthesis blockade alone, and to identify any interactions between these two systems.

## Materials and Methods

Fifteen chronically catheterized fetal sheep between 124 and 136 days' gestation were used in this study. The pregnant ewes were of mixed Western breeds. These experiments were approved by the University of Florida Institutional Animal Care and Use Committee and were performed in accordance with the Guiding Principles for the Care and Use of Animals of the American Physiological Society.

### Surgical Preparation

Aseptic surgery was performed at least 5 days before the start of experiments in each animal. Nine fetal sheep were subjected to catheterization only. Six fetal sheep were subjected to catheterization plus denervation of arterial baroreceptors and chemoreceptors. Fetal hindlimbs were identified and delivered through a small hysterotomy incision near the tip of one uterine horn. As previously described, we

introduced polyvinyl chloride catheters into the tibial artery (.050" ID; .090" OD) and saphenous vein (.030"ID, .050" OD) bilaterally and advanced the tips to the abdominal aorta and inferior vena cava, respectively. After closing the skin incisions in the fetal hindlimb, amniotic fluid catheters were sutured to the fetal skin and the hindlimb returned to the amniotic space. After placement of these vascular and amniotic fluid catheters and closure of the hysterotomy, the fetal head was located, the uterus was incised, and the head was delivered. After a single midline incision in the skin of the neck was made, lingual arteries were identified, ligated, and catheterized with polyvinyl chloride catheters (0.030 in ID, 0.050 in. OD), with the catheter tips advanced retrograde to the lumen of the common carotid arteries. As previously described, this catheterization technique allows measurement of common carotid arterial pressure without interruption of carotid arterial blood flow (Breuhaus and Chimoskey, 1985; Wood et al. 1993). In fetuses subjected to baro- and chemo-denervation, the common carotid arteries and cervical vagosympathetic trunks were carefully exposed on both sides. The vagosympathetic trunks were cut bilaterally. The carotid sinus nerves were identified bilaterally and cut. The walls of the common carotid arteries in this area, as well as the lingual arteries and common carotid arteries extending 0.5-1 cm rostral from the lingual-carotid arterial junction were stripped of all visible nerve fibers. The carotid sinus denervation procedure used in this investigation was shown in a previous study to block the changes in fetal heart rate during hypoxia (Wood et al. 1990). Standard tests of the completeness of denervation were not possible in the present experiments, because the severing of cervical vagosympathetic trunks interrupts the efferent limb of the reflex decrease in heart rate that would be

observed after phenylephrine injection (Wood, 1989; Wood and Rudolph, 1983). After performing these denervations in the fetal neck, the fetal skin was closed, the head was returned to the amniotic cavity, and the uterus was closed.

In some fetuses, we introduced a Swan-Ganz catheter (size 5F) into the saphenous vein of one hindlimb and advanced the tip to the supradiaphragmatic inferior vena cava. In other fetuses, we placed an extravascular balloon occluder around the supradiaphragmatic inferior vena cava using a separate incision through the fourth intercostal space. Extravascular balloon occluders were either purchased (In Vivo Metric, Healdsburg, CA) or fabricated in the laboratory. Occluders fabricated in the laboratory were made from Penrose tubing (0.5 cm diameter), looped around the vena cava (between the diaphragm and right atrium) and connected to polyvinyl chloride tubing. Results from fetuses with intravascular versus extravascular balloon occluders were not distinguishable and therefore were combined. All catheters exited via a small incision in the flank of the ewes.

Ampicillin trihydrate (Polyflex; Aveco Co, Fort Dodge, IA) 500 mg was administered to the fetus via the amniotic fluid and to the mother (500-750 mg) intramuscularly at the time of surgery and again each time the fetus was studied or the catheters were flushed. Ampicillin (500-750 mg) was administered to the mother intramuscularly twice daily for five days after surgery. All catheters were flushed and reheparinized at least once every 3 days.

Experimental Protocol

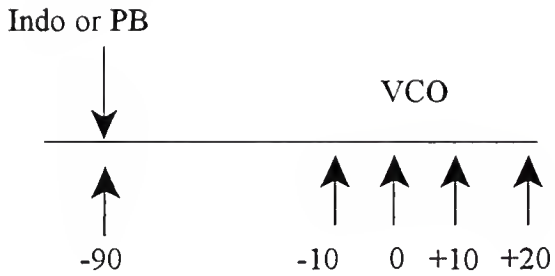



Figure 3-1. Experimental design. In each experiment, indomethacin (Indo) or phosphate buffer (PB) was injected intravenously 90 min before the start of vena caval obstruction (VCO). VCO was achieved for a period of 10 min (between 0 min and 10 min). Fetal blood samples were drawn at -90 min (prior to injection of Indo or PB), at -10 min (prior to the start of the VCO), at 10 min (the end of VCO), and at 20 min (10 min after the end of VCO).

Sheep were transported to the procedure room from their pens within the Health Center Animal Resources Department at least 1 hour before the start of each experiment. Each fetus was studied twice. Experiments consisted of a 90 min pre-occlusion control period (-90 to 0 min), a 10 min occlusion period (0 to 10 min) and a 10 min post-occlusion recovery period (10 to 20 min) (Figure 3-1). In one experiment on each fetus, the vehicle for indomethacin (0.1 M phosphate buffer) was injected intravenously, and in the other experiment 0.2 mg/kg indomethacin (an inhibitor of cyclooxygenase) was injected intravenously 90 min prior to the 10 min period of hypotension. In each experiment, the intravascular or extravascular occluder was completely inflated for 10 min to produce arterial hypotension, which blood pressure was decreased by 50% of normal level.

Blood Sample Handling

Fetal arterial blood samples were drawn from tibial artery catheters (descending aorta) at 90 and 10 min before the start of the period of occlusion, at the end of the 10 min period of occlusion, and 20 min after the start of occlusion. Blood samples (3 ml) were collected into chilled polystyrene tubes containing 150 µl of 0.5 M EDTA. Separate blood samples (1 ml) were collected into chilled polypropylene tubes containing 50 µl 0.5 M EDTA and 40 µg/ml indomethacin for measuring $TxB_2$, 6-keto-$PGF_{1\alpha}$ and $PGE_2$. Tubes were kept on ice until the end of the experiment and then centrifuged for 20 min at 3000 x *g* at 4 ° C. Plasma was separated and stored in separate aliquots at -20 ° C.

Radioimmunoassay (RIA) for Hormone Assays

Plasma adrenocorticotropic hormone (ACTH), cortisol, and arginine vasopressin (AVP) concentrations were measured by specific radioimmunoassay. In ACTH and AVP assays, all plasma samples were run in one assay, and the standard was extracted with plasma to correct for recovery. ACTH was measured using $^{125}$I-ACTH and rabbit anti-ACTH antiserum produced in this laboratory. Before assay, ACTH was extracted from plasma and standard with powdered glass (0.5 ml of 70 mg/ml slurry of glass in 0.5 M phosphate buffer and 0.1% glass extracted bovine serum albumin; Corning Co., Corning, NY). The glass was then washed with buffer and the ACTH eluted from the glass with acid-acetone (1.0 N HCl : 1 acetone). The recovered ACTH (the recovery is about 70-80%) was dried on an evaporator (Savant Instruments, Farmingdale, NY), then frozen at -

20C until assay. The extracts were reconstituted in 500 µl assay buffer (0.5% mercaptoethanol in 0.5 M phosphate buffer with extracted BSA, pH 7.4). The standards in this assay range from 20 to 800 pg/ml. Cortisol was measured using $^{3}$H-cortisol purchased from Amersham Co (Arlington Heights, IL) and rabbit anti-cortisol antiserum produced in Dr. Wood's laboratory. Before assay, cortisol was extracted from plasma using 20 volumes of ethanol. AVP was measured using anti-AVP antiserum also produced in Dr. Wood's laboratory and $^{125}$I-labeled AVP purchased from Amersham (Arlington Heights, IL). Before assay, AVP was extracted from plasma on bentonite (Sigma Co., St. Louis, MO) and eluted from bentonite with acid-acetone. The recovered AVP was dried on an evaporator, then frozen at -20C until assay. The ACTH, cortisol, and AVP assays have been completely described elsewhere (Raff et al. 1991; Wood et al. 1993).

Enzyme-Linked Immunoassay (EIA) for Prostanoids Assays

$PGE_2$, thromboxane $B_2$ ($TxB_2$, a stable metabolite of $TxA_2$), and 6-keto-prostaglandin $F_{1\alpha}$( 6-keto-$PGF_{1\alpha}$, a stable metabolite of $PGI_2$) were measured using enzyme-linked immunoassay kits purchased from Cayman Chemical. Before assay, the prostanoids were extracted from acidified plasma with 6 volumes of ethyl acetate. The recovery using this protocol averages approximately 60%, and the extracted prostanoids diluted parallel to the standard curves. All plasma were run in one assay.

Blood Pressure Recording

Fetal arterial blood pressure and amniotic fluid pressure were measured continuously during the 110 min experiments using a Grass recorder and Statham P23 ID pressure transducers. Not all hemodynamic variables were successfully measured in all experiments. These hemodynamic values were recorded, and analog-to-digital conversions were performed at 2-s intervals using an IBM PC computer. The data collection was achieved using ASYSTANT$^{+}$ software (Asyst Technologies, Rochester, NY). All fetal intravascular pressure were corrected by subtraction of amniotic fluid pressure. One minute means were analyzed statistically.

Statistical Analyses

Changes in the values of fetal hormonal and prostanoids variables over time and between groups were analyzed using two-way analysis of variance (ANOVA) corrected for unequal cell size and for repeated measures in one dimension, time (Winter, 1971). Multiple comparison of mean values was performed using the Student-Newman-Keuls test. The hormonal data were not distributed normally. All ANOVAs performed on hormonal data were calculated after logarithmic transformation to correct for heteroscedasity of the data. A significance level of $P < 0.05$ was used to reject the null hypothesis in all tests. Analyses were performed using SigmaStat software (Jandel Scientific, San Rafael, CA).

## Results

### Prostanoids

The mean values of fetal $PGE_2$, $TxB_2$, and 6-keto-$PGF_{1\alpha}$ concentrations are reported in Table 3-1. In intact fetuses, hypotension significantly increased plasma concentrations of $PGE_2$ (when compared to the preocclusion value), but did not significantly change plasma concentrations of the other measured prostanoids. Indomethacin significantly decreased the plasma concentrations of $PGE_2$, 6-keto-$PGF_{1\alpha}$, and $TxB_2$ in response to vena caval occlusion (Table 3-1).

Table 3-1. Plasma concentrations of prostanoids.

| Time (min) | | Intact | | Denervated | |
|---|---|---|---|---|---|
| | | PB | Indomethacin | PB | Indomethacin |
| -90 min | $PGE_2$ | 105.3 ± 29.7 | 150.9 ± 38.7 | 108.6 ± 53.6 | 77.0 ± 24.3 |
| | $TxB_2$ | 116.8 ± 31.7 | 169.8 ± 39.1 | 62.3 ± 14.3# | 70.0 ± 27.2 |
| | $PGF_{1\alpha}$ | 151.1 ± 68.4 | 159.0 ± 49.4 | 106.1 ± 28.8 | 105.8 ± 33.4 |
| -10 min | $PGE_2$ | 158.3 ± 13.8 | 40.1 ± 4.5 | 98.9 ± 29.2 | 62.8 ± 27.7 |
| | $TxB_2$ | 143.4 ± 26.9 | 132.2 ± 40.3 | 53.7 ± 14.0# | 48.5 ± 16.7 |
| | $PGF_{1\alpha}$ | 196.8 ± 37.8 | 95.0 ± 29.2 | 100.6 ± 26.2 | 100.8 ± 31.0 |
| 10 min | $PGE_2$ | 235.5 ± 35.2*† | 35.1 ± 4.4* | 140.4 ± 43.5 | 60.2 ± 26.5 |
| | $TxB_2$ | 173.3 ± 46.3*# | 105.1 ± 33.1* | 73.4 ± 20.6# | 34.6 ± 11.9 |
| | $PGF_{1\alpha}$ | 275.3 ± 45.0*# | 112.0 ± 32.4* | 166.1 ± 63.7# | 134.0 ± 40.2 |
| 20 min | $PGE_2$ | 298.5 ± 86.6*† | 34.0 ± 5.9* | 107.0 ± 26.9 | 29.2 ± 8.4 |
| | $TxB_2$ | 205.5 ± 73.3* | 107.1 ± 30.7* | 136.8 ± 55.1 | 34.9 ± 11.1 |
| | $PGF_{1\alpha}$ | 230.3 ± 77.9* | 92.1 ± 28.3* | 129.1 ± 41.3 | 97.3 ± 27.5 |

Responses are grouped by the time of vena caval occlusion and intact or denervated and indomethacin or phosphate buffer (PB) treatments. The periods of occlusion were from 0 to 10 min. Prostanoids concentrations were presented as pg/ml ± SE. * indicates statistical significance between the indomethacin and PB treatments groups at $p < 0.05$ (Student's Newman-Keuls). # indicates statistical significance between the intact and denervated groups treated with PB at $p < 0.05$ (Student's Newman-Keuls). † indicates statistical significance when compared within-group to the value at -10 min.

Plasma concentrations of the prostanoids were reduced by denervation of arterial baroreceptors and chemoreceptors. In particular, plasma $TxB_2$ concentrations were significantly lower before and during hypotension in denervated fetuses treated with phosphate buffer. Plasma $PGF_{1\alpha}$ concentrations during hypotension were lower in (phosphate buffer-treated) denervated fetuses than in intact fetuses. Indomethacin did not produce further decreases in plasma prostanoid concentrations in the denervated fetuses.

Cardiovascular Variables

In the intact group (n = 9), vena caval occlusion significantly decreased mean arterial blood pressure from 42.9 ± 2.2 to 24.8 ± 3.7 mm Hg (mean ± SEM) in the fetuses treated with PB; mean arterial blood pressure decreased from 46.5 ± 1.8 to 26.0 ± 3.1 mm Hg in fetuses treated with indomethacin (Fig. 3-2). The effect of the vena caval occlusion on blood pressure was not significantly different in intact *vs.* denervated groups. The values of fetal blood gases and pH are reported in the table 3-2. There are no statistical differences between the intact and denervated groups and phosphate buffer and indomethacin treatment groups.

Table 3-2. Fetal blood gases and pH before indomethacin or PB injections

| | Intact | | Denervated | |
|---|---|---|---|---|
| | PB | indomethacin | PB | indomethacin |
| $PaO_2$, mm Hg | 19.70 ± 1.18 | 18.16 ± 1.18 | 17.94 ± 0.92 | 18.27 ± 2.10 |
| $PaCO_2$, mm Hg | 51.51 ± 1.81 | 45.71 ± 1.94 | 47.43 ± 1.71 | 49.88 ± 2.34 |
| pH | 7.34 ± 0.01 | 7.36 ± 0.01 | 7.37 ± 0.01 | 7.34 ± 0.01 |

Values are means ± SE.

Endocrine Variables

The initial values of ACTH, AVP, and cortisol (before the injection of phosphate buffer or indomethacin) were not significantly different between the intact and denervated groups. Compared with phosphate buffer-treated fetuses in the intact group, indomethacin treatment significantly attenuated the fetal plasma ACTH and AVP reflex responses to vena cava occlusion (Fig. 3-3). Vena caval occlusion increased fetal plasma ACTH concentration significantly from 83 ± 39 to 3611 ± 774 pg/ml, AVP from 4 ± 0.5 to 1079 ± 549 pg/ml, and cortisol from 5 ± 1 to 10 ± 2 ng/ml in fetuses treated with phosphate buffer in intact group; in the indomethacin treatment fetuses, ACTH increased significantly from 156 ± 63 to 1922 ± 674 pg/ml, AVP increased significantly from 5 ± 1 to 226 ± 181 pg/ml, and cortisol from 8±2 to 10 ± 3 ng/ml. After conversion of data to common logarithms, analysis by ANOVA indicated that indomethacin attenuated the ACTH, AVP, and cortisol responses to the vena caval occlusion (significant interaction of time × group in the two-way ANOVA corrected for unequal cell size and for repeated measures in one dimension, time).

The ACTH, AVP, and cortisol responses to the vena caval occlusion were also significantly attenuated in the phosphate buffer-treated denervated fetuses compared to phosphate buffer-treated intact fetuses (significant main effect of group with significant interaction of time × group in the two-way ANOVA). Vena caval occlusion increased fetal plasma ACTH concentration from 201 ± 77 to 456 ± 135 pg/ml, and AVP from 4 ± 0.5 to 16 ± 6 pg/ml in the phosphate buffer-treated denervated fetuses. In contrast to its

effect in intact fetuses, indomethacin had no further effect on the ACTH, AVP, or cortisol responses to vena caval occlusion in the denervated fetuses (no significant main effect of group and time × group interaction). After indomethacin treatment, vena caval occlusion increased plasma ACTH concentration from 282 ± 66 to 1246 ± 560 pg/ml and AVP from 8 ± 3 to 17 ± 6 pg/ml in the denervated fetuses.

## Discussion

The results of this study confirm and substantially extend our previous experiments designed to test the mechanism of the hormonal responses to hypotension in the fetus. First, these results confirm our earlier observations that vena caval occlusion is a stimulus to fetal ACTH, AVP, and cortisol secretion (Wood, 1995b), and that the ACTH and AVP responses are attenuated by arterial baroreceptor and chemoreceptor denervation (Wood, 1989). These results therefore support our earlier conclusions that sinoaortic afferent fibers partially mediate these responses to hypotension. These data extend our earlier observations by demonstrating that the baro- and/or chemo-reflex control of ACTH and AVP secretion is partially dependent upon the endogenous production of prostanoids. There are several important implications of these results which we will address individually.

### ACTH and AVP Responses to Hypotension in the Sheep Fetus Are Not Completely Mediated by Carotid Sinus and Vagal Afferent Fibers

In previous studies, we have demonstrated that carotid sinus denervation attenuated the ACTH and AVP responses to systemic hypotension (produced by vena

caval obstruction) or to carotid arterial hypotension (produced by bilateral carotid occlusion). Sinoaortic denervation impairs the ability of the fetus to maintain arterial blood pressure during progressive hemorrhage (Chen and Wood, 1992). On the other hand, bilateral cervical vagosympathetic nerve section has no apparent effect on the reflex hormonal responses to hemorrhage or on the ability of the fetus to maintain blood pressure when blood volume is reduced (Wood et al. 1989). We therefore strongly suspected that the carotid sinus afferent fibers were more important for reflex hormonal and cardiovascular responsiveness than afferent fibers in the vagosympathetic trunks carrying afferent information from cardiopulmonary receptors. It was possible, although perhaps not likely, that the ACTH and AVP responses to hypotension would be completely eliminated by total (carotid sinus and vagosympathetic nerve section) denervation. The present results prove that the ACTH and AVP responses to hypotension are not completely mediated by neural afferent fibers from these regions.

In the present study, we measured arterial blood gases only at the beginning of each experiment. In a previous study, we found that vena caval obstruction produced no statistically significant changes in fetal $P_aO_2$ or $P_aCO_2$, but did produce moderate decreases in $pH_a$ (approximately 0.04 pH units) (Wood et al. 1982). In another study, we found that such small changes in $pH_a$, on their own, were not sufficient to stimulate either ACTH or vasopressin secretion (Wood and Chen, 1989). Finally, we found that sinoaortic denervation did not alter this pattern of decreasing $pH_a$ and unchanging $P_aO_2$ or $P_aCO_2$ (Wood, 1989). For these reasons, we do not think that the responses that we observe during vena caval obstruction are caused by changes in arterial blood gases, or that baro-

and chemo-denervation alters arterial blood gases during this manipulation. Increases in plasma hormone concentrations can also be caused by hypotension-induced decreases in hormone clearance from plasma. While this can be a complicating factor, even dramatic changes in clearance cannot account for the large changes in plasma concentrations that we have measured.

## ACTH and AVP Responses to Hypotension Are Mediated, in Part, by Endogenous Prostanoids

An important result of the present study is the demonstration that the ACTH and AVP responses to hypotension are partially inhibited by indomethacin in intact fetuses. We and others have previously demonstrated that *exogenous* $PGE_2$ and $TxA_2$-mimetic (U46619) stimulates ACTH secretion (Brooks, 1992; Brooks and Gibson, 1992; Cudd and Wood, 1991; Cudd and Wood, 1993; Wood et al. 1993). It is also well known that in newborn piglets the endogenous production of $PGE_2$, $PGI_2$, and $TxA_2$ is stimulated during induced reductions in cerebral blood flow (Chemtob et al. 1990b). These prostanoid responses are thought to be important for the autoregulation of blood flow when perfusion pressure is changed (Chemtob et al. 1990a). Although the prostanoid generation and release into venous blood is often thought of as originating in the vasculature, it is known that the neurons contain the prostaglandin G/H synthase (Breder et al. 1992) and thromboxane synthase (Husted and Wood, 1998). We designed the present experiments to test the hypothesis that endogenous production of prostanoids, whether in vasculature

or in neurons, might partially mediate the reflex hormonal responses to hypotension. The results indicate that this hypothesis was correct.

The results reveal an interaction between the afferent fibers and the endogenous prostanoids. Indomethacin had a statistically significant effect only in the intact fetuses. The ACTH and AVP responses were attenuated by the denervation procedure, but were not further attenuated by indomethacin. This suggests that the action of the prostanoids must be on the afferent pathways or on a central element which is activated by the afferent pathways. In other words, this selective action appears to rule out an effect solely on the anterior or posterior pituitary or on the nerve terminals of the median eminence, because an action on the so-called "final common pathway" would attenuate the response in both groups. The results therefore demonstrate that indomethacin is not simply a "nonspecific" inhibitor of ACTH and AVP secretion.

We and others have found that exogenous prostanoids may stimulate hormonal and hemodynamic responses by affecting the neuronal processing within the central nervous system. These studies have focused mainly on $PGE_2$ and thromboxane $A_2$ ($TxA_2$). Infusions of $PGE_2$ into the carotid arteries of conscious adult sheep increase heart rate and blood pressure (Breuhaus and Chimoskey, 1983; Breuhaus and Chimoskey, 1985; Breuhaus et al. 1989) and ACTH secretion (Cudd and Wood, 1991). This effect is not attenuated by carotid sinus baro- or chemo-denervation (Cudd and Wood, 1993) and therefore is likely to be a direct effect on the brain. Infusions of $PGE_2$ also stimulate increases in fetal blood pressure and heart rate (Cudd and Wood, 1992; Cudd and Wood, 1993). Intracerebroventricular (Brooks and Gibson, 1992) or intravenous (Young and

Thorburn, 1994) infusion of $PGE_2$ can stimulate ACTH and cortisol secretion in fetal sheep, and treatment with indomethacin decreases ACTH release (Thompson and Hedge, 1978). In addition to its effect on the brain, $PGE_2$ has a direct effect on the fetal sheep pituitary gland by enhancing AVP-stimulated, but not CRH-stimulated, ACTH secretion from dispersed fetal anterior pituitary cells in culture (Brooks, 1992). $TxA_2$ stimulates increases in arterial blood pressure and heart rate and in the rate of ACTH secretion in both adult and fetal sheep (Cudd, 1997).

The influence of prostanoids on AVP secretion has been investigated in adult dogs and rats and in newborn piglets. Intraventricular administration of $PGE_2$ (Hoffman et al. 1982; Yamamoto et al. 1976) or $PGD_2$ (Brooks et al. 1986) stimulates AVP secretion. Indeed, the stimulation of AVP secretion in response to intracerebroventricular injections of angiotensin II or acetylcholine is attenuated by indomethacin (Inoue et al. 1990; Inoue et al. 1991), suggesting that central pathways affecting AVP secretion by hypothalamic magnocellular neurons are dependent upon prostanoid generation. AVP responses to hypertonic saline are significantly inhibited by prior administration of meclofenamate, indomethacin, or of flunixin meglumin (Brooks et al. 1986; Hoffman et al. 1982; Patel et al. 1997). The effect of endogenous prostanoids on the AVP responses to hemorrhage were somewhat more variable. Meclofenamate, but not indomethacin, attenuated the AVP response to hemorrhage in pentobarbital-anesthetized dogs (Brooks et al. 1984). Indomethacin augmented the AVP response to hemorrhage in morphine sedated and urethane-chloralose-anesthetized dogs (Brooks et al. 1984). These interesting experiments suggest that there might be a differential effect of indomethacin and

meclofenamate on the relative degrees of inhibition of the prostanoid biosynthetic enzymes (cyclooxygenase, thromboxane synthase, etc.). Or, perhaps, there might be an effect of anesthesia on the degree to which prostanoids influence AVP secretion.

We measured plasma concentrations of $PGE_2$, 6-keto-$PGF_{1\alpha}$, and $TxB_2$ before and after indomethacin injection to confirm our assumption that the production of prostanoids would be reduced. Overall, indomethacin did effectively reduce the plasma concentrations of all three prostanoids, although the effect was more pronounced in the intact fetuses. The denervated fetuses had lower plasma prostanoid concentrations initially, and suppression was therefore less pronounced. Although we used plasma concentrations as an index of overall prostanoid production, it is important to emphasize that these compounds are most likely not acting as hormones, and that the local concentrations within the brain were unaccessible to us with the present experimental design. We are confident that the circulating (plasma) concentrations of $PGE_2$, and probably other prostanoids as well, are too low to stimulate ACTH secretion. Cudd and Wood have found that high rate infusions of $PGE_2$ into the carotid artery stimulate ACTH secretion in both fetal and adult sheep (Cudd and Wood, 1991; Cudd and Wood, 1992). However, when $PGE_2$ is infused at rates which increase carotid arterial plasma concentrations within the physiological range, no effect on ACTH was measured (Cudd and Wood, 1991; Cudd and Wood, 1992). We believe that the response to $TxA_2$ is analogous. We demonstrated that infusions of U46619 (a stable $TxA_2$ mimetic) into the carotid arterial blood of fetal sheep stimulates ACTH secretion, whereas similar infusions into the venous blood (and therefore diluted by the entire cardiac output) does not (Wood et al. 1993). $TxA_2$

therefore acts at the brain to stimulate ACTH secretion. Because we used a stable mimetic drug (with a relatively long half-disappearance time in blood), we could not assess the physiological significance of the concentrations of $TxA_2$ naturally occurring in plasma. However, $TxA_2$ is very unstable in aqueous solution and would be unlikely to reach the brain in physiologically meaningful amounts.

One interesting result of the present experiments (although probably not related to the ACTH and AVP secretion) is the increase in plasma prostanoid concentrations during the period of hypotension. This release of prostanoids into the plasma is reminiscent of the $PGE_2$ and $PGI_2$ production by the umbilical-placental vasculature in response to angiotensin II (Yoshimura et al. 1990). Indeed, it is impossible to identify the source of these circulating prostanoids: they could originate from any of the systemic or pulmonary vascular beds. Nevertheless, it is tempting to speculate that the prostanoid release into plasma is secondary to the reflex vasoconstriction during hypotension. If so, the reduction in prostanoid concentration in the denervated animals would be explained by a lower level of vasomotor tone in these animals (Jacob et al. 1991).

In conclusion, the results of this study demonstrate that the endogenous production of prostanoids modulates the reflex hormonal responses to hypotension in late-gestation fetal sheep. We speculate that the prostanoids are generated either on the afferent nerves subserving arterial baroreceptors and chemoreceptors, or that they are generated within the nucleus of the tractus solitarius or other sites within the central nervous system receiving afferent input from baroreceptors and chemoreceptors.

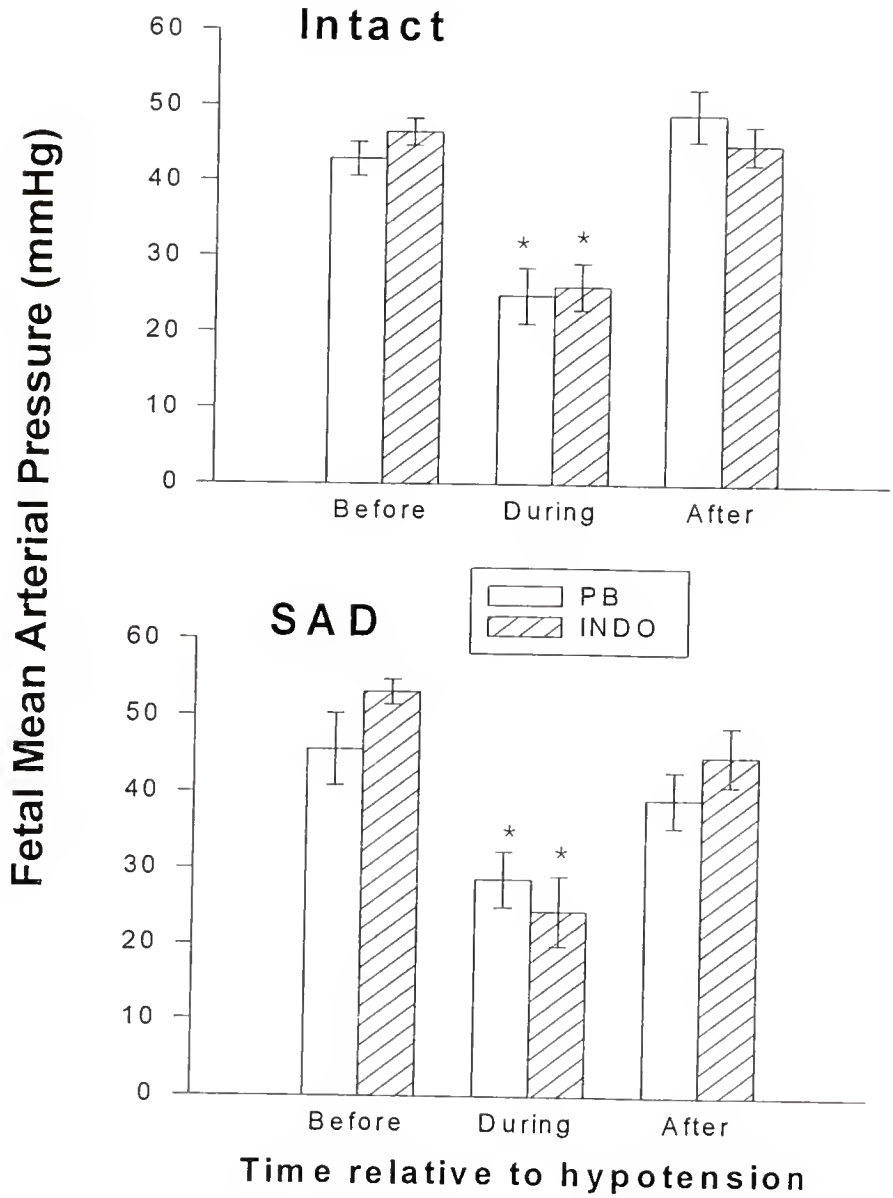



Figure 3-2. Fetal mean arterial blood pressure (MAP) before, during and after a 10-min period of vena caval obstructions in the intact (top panel) and denervated (bottom panel) fetuses. There were no significant differences of MAP between intact and denervated fetuses or between phosphate buffer or indomethacin treated groups. Mean values are accompanied by vertical bars representing 1 SEM. *$p < 0.05$ indicates that VCO significantly decreased fetal MAP.

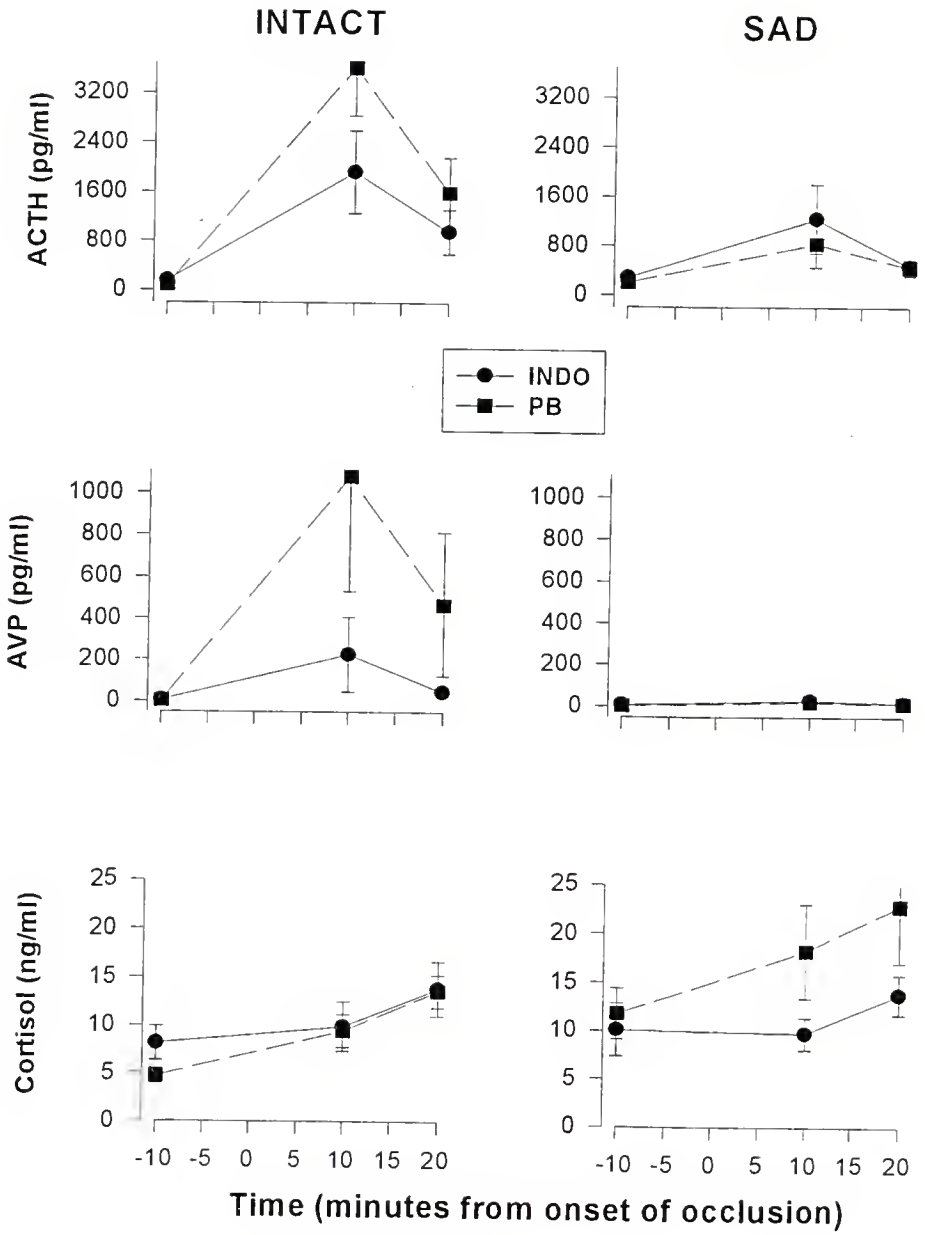



Figure 3-3. Fetal plasma ACTH, AVP and cortisol concentrations before, during, and after a 10-min period of vena caval obstruction in the intact and denervated (SAD) fetuses. The period of hypotension is from 0 min to 10 min. Mean values of hormones in phosphate buffer- (■, dashed lines) and indomethacin- (●, solid lines) treated fetuses are accompanied by vertical bars representing ±1 SEM. Two-way ANOVA revealed a significant interaction of time × treatment ($p < 0.05$) in the intact but not in the denervated fetuses.

# CHAPTER 4
# PROSTAGLANDIN ENDOPEROXIDE SYNTHASE-2 IS THE MAJOR CONTRIBUTOR TO PROSTANOID BIOSYNTHESIS DURING CEREBRAL HYPOPERFUSION IN LATE-GESTATION FETAL BRAIN TISSUES

## Introduction

Prostaglandin endoperoxide synthase (PGHS) catalyzes the oxygenation (cyclooxygenase) of arachidonate to the prostaglandin endoperoxide $PGG_2$ and the reduction (peroxidase) of $PGG_2$ to $PGH_2$. $PGH_2$ is further converted by specific synthases (isomerases) or reductases to the major biologically active prostanoids $PGD_2$, $PGE_2$, $PGF_{2\alpha}$, $PGI_2$, and thromboxane $A_2$ ($TxA_2$) (Smith et al. 1991). A unique feature of PGHS is the autoinactivation of the enzyme following the conversion of $PGG_2$ to PGH2 (Hemler and Lands, 1980b).

Two different isoforms of PGHS were confirmed at the protein (Habib et al. 1993) and mRNA levels (O'Neill and Ford-Hutchinson, 1993). There are two distinct genes which encode for PGHS-1 and PGHS-2. PGHS-1 is the isoenzyme which was first purified from bovine and ovine microsomes and is a homodimer consisting of two subunits each of approximately 70 kilodaltons (Samuelesson et al. 1978). PGHS-1 is constitutively expressed in most tissues. It is linked to basal synthesis of prostanoids during cell differentiation and it is not suppressed by corticosteroid hormones (DeWitt, 1991). Recently, however, it has been shown that PGHS-1 can be slightly induced, contributing

to the up-regulation of prostanoid biosynthesis during monocyte differentiation (Hoff et al. 1993). The other isoenzyme, PGHS-2, is an inducible, transient form of PGHS. It can be induced by a variety of stimuli such as mitogens, growth factors (Kujubu et al. 1992), inflammatory agents (Lee et al. 1992), hormones (Wong and Richards, 1992) and physiological activity (Ymagata et al. 1993), suggesting its involvement in physiological responses.

The two PGHS isoforms differ in tissue distribution. Breder et al. (1992, 1995) have demonstrated that the distribution of PGHS-2 in the central nervous system was quite different from PGHS-1. One study demonstrated that PGHS-2 is the predominant form of prostaglandin endoperoxide synthase in the brain of newborn piglet (Peri et al. 1995). PGHS-1 and PGHS-2 mRNAs are expressed equally in most human tissues except that PGHS-2 mRNA has a higher level in lung tissues (O'Neill and Ford-Hutchinson, 1993).

Thromboxane synthase catalyzes the formation of thromboxane $A_2$ from prostaglandin $H_2$. The enzyme has been purified from lung and platelets. Western blot studies indicated that the immunoreactive thromboxane synthase molecular weight ranges from 46 to 130 KD (Wood et al. 1997), depending on the source tissue and analytical method for determination. This enzyme is widely distributed in the brain (Wood et al. 1997).

Prostaglandins are local hormones which are synthesized by virtually all mammalian tissues (Smith, 1987) and act in an autocrine and/or paracrine fashion. These bioactive lipids have important roles in regulating several neurological (Smith, 1987;

Hayaishi, 1991), cerebral hemodynamic functions (Wolfe and Coceani, 1979) and cardiovascular activities (Siren, 1982; Chemtob et al. 1990b). Our previous studies demonstrated that endogenous prostanoids had significant effects on modulating adrenocorticotropic hormone (ACTH) and arginine vasopressin (AVP) secretions from fetal sheep during moderate arterial hypotension (Tong et al. 1998). The present studies were conducted to determine the mechanism by which cerebral hypoperfusion enhances prostanoid secretion by fetal brain tissues. Specifically, we investigated the ability of cerebral hypoperfusion to modify levels of the constitutive (PGHS-1), inducible (PGHS-2) isoforms of PGHS, and thromboxane synthase in brain tissues.

## Materials and Methods

### Materials

Polyclonal antibodies specific to PGHS-1 and PGHS-2 were purchased from Oxford Biomedical Research, Inc. (Oxford, MI). Polyclonal antibody specific to thromboxane synthase, and arachidonate, $PGH_2$ and $PGE_2$, $TxB_2$ enzyme immunoassay kits were purchased from Cayman Chemical (Ann Arbor, MI). Diethyldithiocarbamic acid (DEDTC) were purchased from Sigma Chemical Co. (St. Louis, MO). Protein assay kits, 10% SDS-polyacrylamide ready gels and electrophoretic reagents, nitrocellulose membranes, and filter papers were purchased from Bio-Rad (Richmond, CA). Rainbow molecular weight markers and horseradish peroxidase-conjugated anti-rabbit IgG antibodies were purchased from Amersham (Arlington Heights, IL). Western blot chemiluminescence reagent were purchased from DuPont NEN (Boston, MA).

Animals

These experiments were approved by the University of Florida Institutional Animals Care and Use Committee and were performed in accordance with the Guiding Principles for the Care and Use of Animals of the American Physiological Society. Time dated pregnant ewes were of mixed Western breeds obtained from the Institute of Food and Agricultural Sciences at University of Florida. Fetal sheep between 124 and 136 days' were used in this study.

One group of fetuses (n = 5) was studied as intact (control) fetuses; the second group of fetuses (n = 5) was studied after carotid sinus denervation (experimental group). The experimental group was subjected to a 10-min period of cerebral hypoperfusion produced by occlusion of the brachiocephalic artery, performed using an extravascular occluder (In Vivo Metric, Healdsburg, CA).

The fetal sheep were anesthetized using 1.5% halothane. Lingual arteries were catheterized bilaterally for measurement of blood pressure, blood gases, and plasma concentrations of prostaglandins in arterial blood. Using our previously described techniques (Wood, 1989), bilateral carotid sinus denervation was performed on the five fetuses in the experimental group to remove the confounding effect of baro- and chemoreflexes. Next, an extravascular balloon was placed around the brachiocephalic artery, exposed using an incision in the left second intercostal space. The sagittal sinus was catheterized to allow sampling of blood at this site for measurement of plasma prostaglandin concentrations in venous blood. Microdialysis probes (CMA Corporation,

size CMA-10) were inserted in brain stem and hypothalamus region for measurement of prostaglandins in interstitial fluid at these sites.

Animals were allowed to stabilize for at least 2 h before starting experiments. The studies consisted of 10-min preocclusion, 10-min occlusion and 10-min postocclusion periods. Size 8 to 10 occluder was used dependent on the animal size. The occlusion of the brachiocephalic artery was complete achived by saline inflatation. Blood samples (3 mL) and microdialysate (flow rate = 160 µL/min) were collected during the 3 periods. Blood samples were kept chilled on ice and plasma was stored at -80° C until assay of prostaglandins.

Tissue Collection

The fetuses were sacrificed 30 min after hypotension using an overdose of pentobarbital. For Western blot and enzyme activity assay studies, fetal hypothalamus, brainstem, cerebellum, hippocampus and cerebral cortex tissues were quickly removed, and snap-frozen in a dry ice and acetone bath and stored at -80° C.

Measurement of $PGE_2$ and $TxB_2$

$PGE_2$ and $TxB_2$ from blood samples and produced in the incubates were measured by enzyme-linked immunoassay (EIA). All incubation medium samples were centrifuged at 2500 x g for 25 min at 4° C and the supernatants were collected for prostanoids assay. This assay has been described in chapter 3.

PGHS Activity Assay

The microsomal PGHS activity (PGHS-1 and PGHS-2) was determined by measuring $PGE_2$ production under initial velocity conditions (Wimsatt et al. 1993). Approximately 1 g frozen tissues were homogenized in 10 ml of homogenization buffer (50 mM Tris, pH=8.0, 0.25 M sucrose, 2 mM EDTA, 1 mM diethyldithiocarbamate) for two 30-sec bursts on ice using a homogenizer. The homogenates were centrifuged at 2200 x g at 4° C for 10 min, then supernatants were centrifuged at 100,000 x g for 60 min at 4° C. The pellet was washed twice with 1 ml reaction buffer (50 mM Tris with 2 mM EDTA, 1 mM diethyldithiocarbamate) and resuspended in 2 ml of this same buffer containing 7.5 µl Tween 20/ml. The suspension was further dissociated using a drill-mounted Teflon-glass homogenizer at moderate speed. The microsome preparation was kept on ice and assayed immediately.

The reaction mixture contained 200 µM free arachidonate, 1 mM hematin, 5 mM and enzyme preparation in a final volume of 250 µl incubated in a water bath at 38° C for 15 sec. The reaction was terminated by the addition of 200 µl 2.65 mM $SnCl_2$ in 50 mM HCl made up immediately before use. The reaction mixture without enayme preparation was for control. All reaction tubes were stored at -80° C until assayed for $PGE_2$.

Thromboxane Synthase Activity Assay

The microsomal thromboxane synthase activity was determined by measuring the ability to convert $PGH_2$ to $TxB_2$ (Shen and Tai, 1986). The tissues were homogenized for

six 20-sec bursts on ice with 3 vol (W/V) of 25 mM Tris-HCl buffer (pH 7.5), then the homogenates were centrifuged at 8,000×g for 30 min at 4° C. The supernatants were centrifuged again at 100,000×g for 1 hr at 4° C. Then the microsome pellets were resuspened in 1/4 vol (v/w) of 25 mM Tris-HCl buffer (pH 7.5).

Thromboxane synthase is a membrane-associated enzyme for which the membrane needs to be solubilized before assay. Therefore, the microsomal preparations were solubilized by stirring the microsomal fraction with solubilization buffer (25 mM Tris-HCl, 20% glycerol, 0.2 mM DTT, 2 mM EDTA, 2% lubrol-PX) at 4° C for 1hr, and the mixtures were centrifuged for 1 hr at 100,000×g. The reaction mixture contained 10 $\mu$M $PGH_2$ and enzyme preparation in a final volume of 200 $\mu$l of 50 mM Tris-HCl buffer (pH 7.5). The reaction mixture without enzyme preparation was for control. All reaction tubes were stored at -80° C until assayed for $TxB_2$.

Western Blot of PGHS-1, PGHS-2 and Thromboxane Synthase

Approximately 200 mg frozen tissues were homogenized in 1 ml of Laemmli buffer (0.5 M Tris-HCl, 5% glycerol, 10% sodium dodecyl sulfate, and 2.5% 2-β-mercaptoethanol) (Laemmli, 1970) for two 30-sec bursts on ice using a homogenizer. The homogenates were centrifuged at 15,000 x g at 4° C for 30 min and the supernatants were stored at -40° C. Total protein concentration of the supernatant was determined by using the Bradford method (Bradford, 1976) and bovine serum albumin as the standard.

The supernatants were boiled in sample loading buffer for 5 min before loading on 10% SDS-polyacrylamide gels. An equal amount of protein was loaded in each lane.

Samples from control and experimental group were run on the same gel. The proteins were electrophoretically transferred to nitrocellulose membranes, and the nonspecific binding sites on the membranes were blocked with blocking buffer containing non-fat milk (15% for PGHS-1, 10% for PGHS-2 and 7.5% for thromboxane synthase respectively), and 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1% Tween 20 for 1h. The membranes were incubated in blocking buffer containing PGHS-1, PGHS-2 and thromboxane synthase specific polyclonal rabbit antibodies (dilution of 1:100, 1:1000 and 1:3000 respectively) for 1h, then incubated in horseradish peroxidase-conjugated anti-rabbit IgG antibodies in blocking buffer for 1h. Finally, the immunoreactive bands were visualized by a Western blot chemiluminescence reagent (DuPont NEN, Boston, MA) and analyzed by densitometry. The ovine PGHS-1 or PGHS-2 standard was used in each experiment to confirm the right molecular weight size. Each experiment was performed three to five times using different homogenates.

Statistical Analysis

Data were analyzed by paired Student's *t* test, and significance was established at $p < 0.05$. Data are expressed as mean ± SEM. Statistical procedures were accomplished using SigmaStat (Jandel Scientific, San Rafael, CA).

# Results

## Blood Pressure and Blood Gases

Brachiocephalic occlusion decreased carotid arterial blood pressure by approximately 56%. Mean arterial blood pressure decreased from 40.6 mm Hg to 17.7 mm Hg, then returned to baseline levels after the occlusion ($p < 0.01$). Hypotension did not affect arterial pH, $P_aO_2$, or $P_aCO_2$ (Table 4-1).

Table 4-1. Fetal mean blood pressure and blood gases during the experiment

| | Before | During | After |
|---|---|---|---|
| MAP (mm Hg) | 40.6 ± 3.5 | 17.7 ± 2.2 | 34.3 ± 2.8 |
| $Pao_2$ (mm Hg) | 27.80 ± 1.91 | 29.08 ± 1.59 | N/D |
| $Paco_2$ (mm Hg) | 64.40 ± 2.05 | 64.93 ± 3.58 | N/D |
| pH | 7.23 ± 0.01 | 7.24 ± 0.01 | N/D |

Values are means ± SE. N/D: not determined.

## Prostanoid Production in the Fetal Brain

Plasma thromboxane $B_2$ ($TxB_2$) concentration in sagittal sinus blood increased significantly after the onset of hypotension and remained high in the 10-min recovery period. The concentration of $TxB_2$ was higher in sagittal sinus plasma than in the arterial plasma. Plasma prostaglandin $E_2$ ($PGE_2$) concentration did not change during hypotension and there was no difference between the plasma concentrations in arterial and sagittal sinus blood (Fig. 4-1). In the microdialysate, $TxB_2$ concentrations in hypothalamus and

brainstem decreased during hypotension then returned to pre-hypotension concentrations ($p < 0.05$). $PGE_2$ concentration in brainstem and hypothalamus microdialysates increased in response to hypotension although this response was statistically significant only in the brainstem (Fig. 4-2; $p < 0.05$).

PGHS-1 and PGHS-2 Protein Expression and Distribution in Brain Tissues and PGHS Enzymatic Activity

To examine the effect of carotid sinus denervation and cerebral hypoperfusion on PGHS levels in fetal brain, Western blot analysis was performed on the constitutive isoform of PGHS-1 and inducible isoform of PGHS-2 in intact and experimental brain tissues. Figure 4-3 (top panel) shows that the immunoreactive PGHS-1 protein (70 kDa) was expressed equally in all regions of the brain and was not affected by the hypotension. The results of these experiments demonstrated no effect of hypotension on immunoreactive PGHS-1 protein levels (Fig. 4-3 bottom panel).

The expression of the immunoreactive PGHS-2 protein was relatively region-specific. Immunoreactive PGHS-2 was detected as a band of 72 kDa and, in some tissues, a band of about 140 kDa. We suspect that this band was a homodimer which may be found in fetal tissues. We observed minor doublet bands of 46 kDa which are likely to be proteolytic fragments of the intact enzyme (data not shown). The 72 kDa molecular weight of PGHS-2 was expressed in higher abundance in brainstem and hippocampus, but less in hypothalamus, cortex, and cerebellum (Fig. 4-4 top panel). Hypotension and denervation increased 72 kDa PGHS-2 protein abundance in all regions (Fig. 4-4 bottom

panel). The expressions of the immunoreactive 140 kDa of PGHS-2 were variable in different brain regions (Fig. 4-5 top panel), but were increased significantly in all regions by hypotension and denervation (Fig. 4-5 bottom panel; $p < 0.05$). There was no significant effect on the lower molecular weight fragments.

The microsomal PGHS activity assay showed that activity decreased significantly in all regions of the brain by hypotension and denervation compared to the intact control ($p < 0.05$) (Fig. 4-6).

Thromboxane Synthase Protein Expression and Distribution in Brain Tissues and Thromboxane Synthase Enzymatic Activity

Western blot analysis was used to test the effect of carotid sinus denervation and cerebral hypoperfusion on the protein expression of thromboxane synthase. Fig. 4-7 (top panel) shows that the expression of the major band on 46 kDa immunoreactive thromboxane synthase protein was variable in the different brain regions. The immunoreactive enzyme was most abundant in brainstem, and more abundant in hippocampus, hypothalamus and cerebellum, than in cerebral cortex. Apparent increases in thromboxane synthase protein levels in response to hypotension were not statistically significant (Fig. 4-7 bottom panel). Microsomal thromboxane synthase activity was not affected by hypotension in the different brain tissues (Fig. 4-8).

## Discussion

The present study was designed to test the hypothesis that high prostaglandin levels in fetal brain during cerebral hypoperfusion may be due to increased PGHS-2

activity and expression. It has been found that prostanoid concentrations in the brain changed during ischemia (Stevens and Yaksh, 1988; Hanamura et al. 1989). Studies in adult animals suggested that during ischemia, the changes in prostanoid concentrations in the brain were important for cerebrovascular control (Dempsey et al. 1986; Chen et al. 1986; Hallenbeck and Furlow, 1979; Shohami et al. 1982). Chemtob et al. (1990c) demonstrated a relationship between changes in sagittal sinus prostanoid concentrations and changes in cerebral blood flow (CBF), suggesting that autoregulation of cerebral blood flow is in part dependent upon local production of prostanoids. Our previous studies demonstrated that endogenous prostanoids modulated the adrenocorticotropic hormone (ACTH) and arginine vasopressin (AVP) responses to moderate hypotension fetal sheep. Both of these hormonal systems are involved in the homeostatic control of blood pressure in fetal animals (Tong et al. 1998).

Our experiments demonstrated that, in the sheep fetus, reduction of cerebral perfusion pressure increases the production of prostanoids, both in the blood draining the brain and in the extracellular fluid in the brain. In this study, blood pressure was decreased approximately 60% by brachiocephalic trunk occlusion to reduce the blood flow to the brain. In separate experiments, we used colored microspheres to demonstrate that brachiocephalic occlusion decreased cerebral blood flow dramatically (unpublished data). In the present experiments, we found that the concentration of thromboxane $B_2$ (the stable metabolite of $TxA_2$), a cerebral vasoconstrictor (Dempsey et al. 1986), increased significantly in sagittal sinus blood *vs* arterial blood during and 10 min after hypotension. But the concentration of prostaglandin $E_2$, a cerebral vasodilator, was not affected by the

hypotension and there was no difference between the arterial and sagittal sinus blood. The elevated plasma levels of $TxA_2$ in sagittal sinus blood during hypotension was consistent with finding in other laboratories (Chemtob et al. 1990c). Chemtob et al. (1990a) suggested that the increase in $TxB_2$ concentration in response to reduced cerebral blood flow was consistent with thromboxane contributing to the lower limit of CBF autoregulation in newborn piglets. Our present results are also consistent with this hypothesis.

Our microdialysis data demonstrated that thromboxane $B_2$ concentrations in the hypothalamus and brainstem microdialysates decreased during hypotension, and returned to normal level after hypotension. But prostaglandin $E_2$ concentrations in brainstem and hypothalamus microdialysate increased during and 10 min after the hypotension (Fig. 4-2). The results of the present study are consistent with the notion that $TxA_2$ may contribute to cerebrovascular control during hypotension and cerebral hypoperfusion. Prostaglandin $E_2$ may be responsible for mediating the hormonal secretions by acting on the secretory neurons in the brain via a autocrine and/or paracrine mechanism. Our speculation concerning dual (cerebrovascular and neuromodulatory) effects of brain prostanoids was based on observations made by others using immunocytochemistry, Western blot and Northern analysis (Breder et al. 1992; Breder et al. 1995; Peri et al. 1995). Peri et al. (1995) demonstrated that the immunoreactive PGHS-1 and PGHS-2 protein and mRNA expressed in newborn and juvenile pig cerebral microvasculature and brain. Breder et al. (1992, 1995) showed that immunoreactive PGHS-1 and PGHS-2 were in neurons and microvasculature in the brain. We cannot distinguish the source of

immunoreactive PGHS protein in our study, although we speculate that the increased prostanoid in sagittal sinus blood was released from cerebral microvasculature, and the increased prostanoid in brain microdialysate was probably released from neurons containing PGHS.

PGHS undergoes "suicide" inactivation during catalysis (Smith and Lands, 1971; Smith and Lands, 1972). Incubation of cyclooxygenase with arachidonic acid results in loss of cyclooxygenase but not peroxidase activity, concurrent with $PGH_2$ production. The chemical basis for inactivation is unknown. Degradation or modification of the heme prosthetic group could account for inactivation (Hemler and Lands, 1980; Chen et al. 1987). Another possibility is that the PGHS gene is an "immediate early gene", its translation product may act to inhibit the transcription of the PGHS gene (Smith et al. 1991). It is important to note that inactivation of PGHS has been demonstrated *in vivo* as well as *in vitro*, indicating that it may indeed be a physiologically relevant process (Lapetina and Cuatrecasas, 1979; Kent et al. 1983). Balancing inactivation is the gene level expression of the protein. Thromboxane synthase is also a "suicide" enzyme (Jones and Fitzpatrick, 1990). Inactivation of thromboxane synthase is a mechanism-based process and the enzyme heme prosthetic group appears to be affected.

Ovine PGHS-2 is more resistant to trypsinization than PGHS-1 (Wimsatt et al. 1993). Our results showed that PGHS-1 was measured as a 70 kDa molecular weight in fetal tissues. Immunoreactive PGHS-2 was expressed as a 140 kDa and 72 kDa molecule in various fetal brain tissues. We speculated that the 140 kDa exists as a homodimer of 72 kDa PGHS-2. Some lower molecular weight proteolytic fragments appeared in the

Western blot. Various factors can affect the susceptibility to enzyme digestion. This difference could result from increased glycosylation and glycosylation sites on the enzyme (Hla and Neilson, 1992). Alternatively, PGHS-2 may exist with a large amount of the enzyme in the heme-bound state, as heme binding can inhibit trypsin degradation of the enzyme (Kulmacz and Lands, 1982). Existence of the enzyme in its unoxidized state is also more susceptible to cleavage by trypsin, suggesting that PGHS-2 might be less readily oxidized than PGHS-1 (Chen et al. 1987). Purified PGHS protein has a molecular weight of 70 kDa when analyzed on denaturing electrophoresis gels. However, when purified PGHS is applied to a gel filtration column, it elutes as a homodimer at an apparent molecular weight of 140 kDa (Hemler et al. 1976). Gel filtration of PGHS can be carried out in buffer containing 6 N guanidine.HCl to interrupt non-covalent interactions, but the purified protein still elutes as a homodimer under these conditions (Ogino et al. 1978). The clipped enzyme retains its molecular mass of 140 kD in solution and can still be acetylated by aspirin (Chen et al. 1987; Chen and Marnett, 1989). Overall, trypsin-digested PGHS appears to behave just like native PGHS, except for its diminished capacity for peroxidase activity. Limited digestion of PGHS into fragments of 33 kD and 38 kD also has diminished peroxidase activity whereas the cyclooxygenase activity persists (Chen et al. 1987; Dildy et al. 1994).

Western blot and enzyme activity assay of PGHS demonstrated that a significant quantitative difference of the two isoforms was noted in different brain regions. In contrast to the newborn piglet (Peri et al. 1995), PGHS-1 was predominant in the intact animals and was not appreciably increased by cerebral hypoperfusion. PGHS-2

immunoreactivity was increased by cerebral hypoperfusion while PGHS enzyme activity was decreased, suggesting that PGHS-2 is responsible for the brain prostanoids production when the fetus suffered from cerebral ischemia. Thromboxane synthase protein levels and activity were not altered by cerebral hypoperfusion and carotid sinus denervation in this study.

Prostaglandins have been shown to exhibit neuroprotective properties (Gazevieille et al. 1994). It is likely that their increased levels during hypotension may provide protection to the fetal brain from low oxygen tension (Dildy et al. 1994) and the risk of hypoxic brain injury increases. Since PGHS-2 is induced rapidly, it would be suited for such a temporary but important role in response to stress.

In summary, this study demonstrates that in late-gestation fetal sheep: 1) prostanoids are produced in response to cerebral hypoperfusion; 2) the increase in the production of prostanoids responses to cerebral hypoperfusion is associated with the decrease in activity of, and therefore the "suicide" inactivation of, prostaglandin endoperoxide synthase, and 3) PGHS-2 is the predominant form of prostaglandin endoperoxide synthase whose synthesis is induced by cerebral hypoperfusion in the fetal brain. We concluded that PGHS-2 is likely the main contributor to the brain prostaglandin levels during cerebral hypoperfusion in the fetuses.

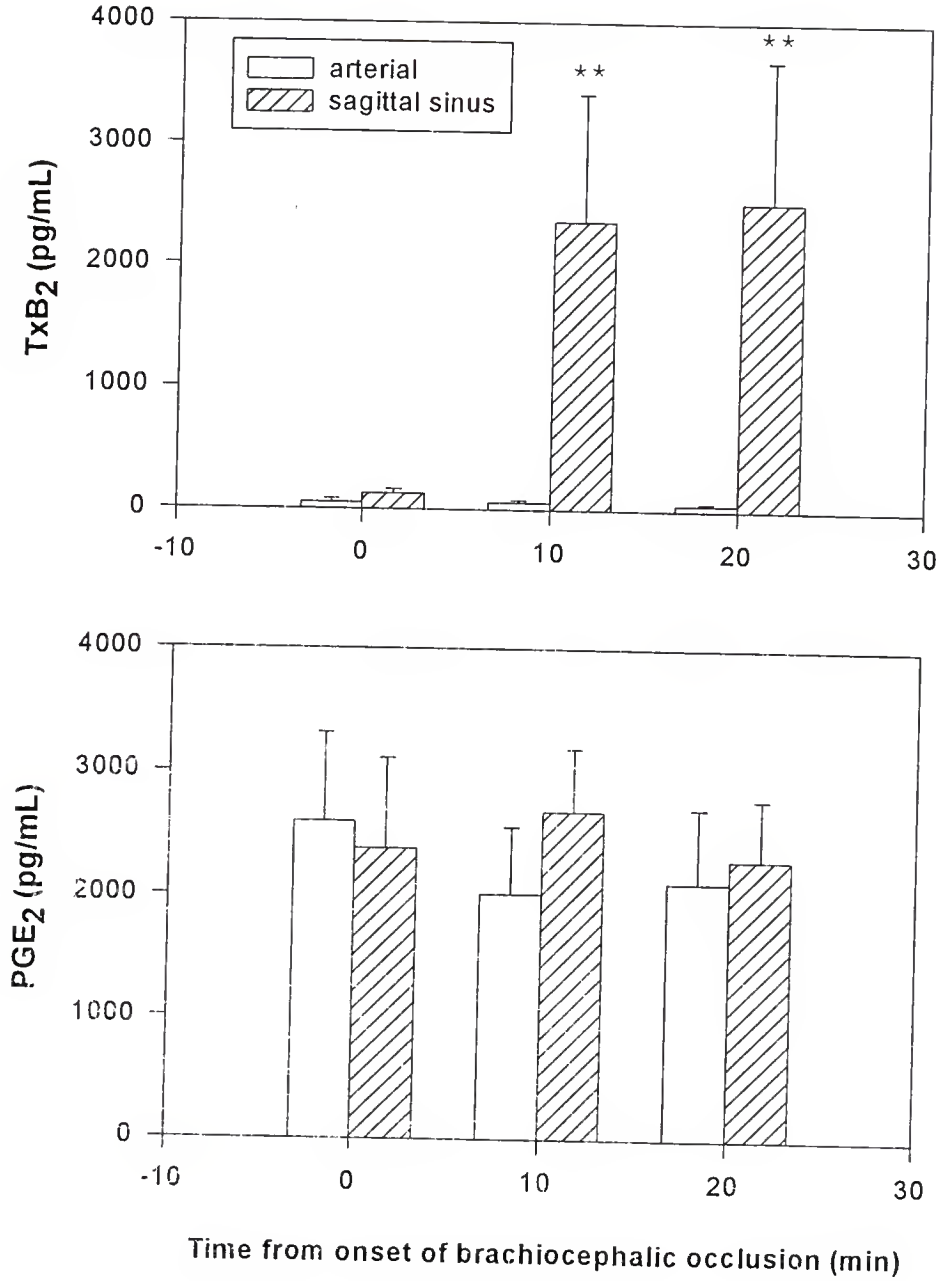



Figure 4-1. $TxB_2$ and $PGE_2$ concentrations (mean ± SE) in fetal arterial (open bar) and sagittal sinus plasma (shaded bar) before, during and 10 min after a 10-min period of brachiocephalic artery occlusion. ** $p < 0.01$, indicates difference from blood sampling sites. N=5.

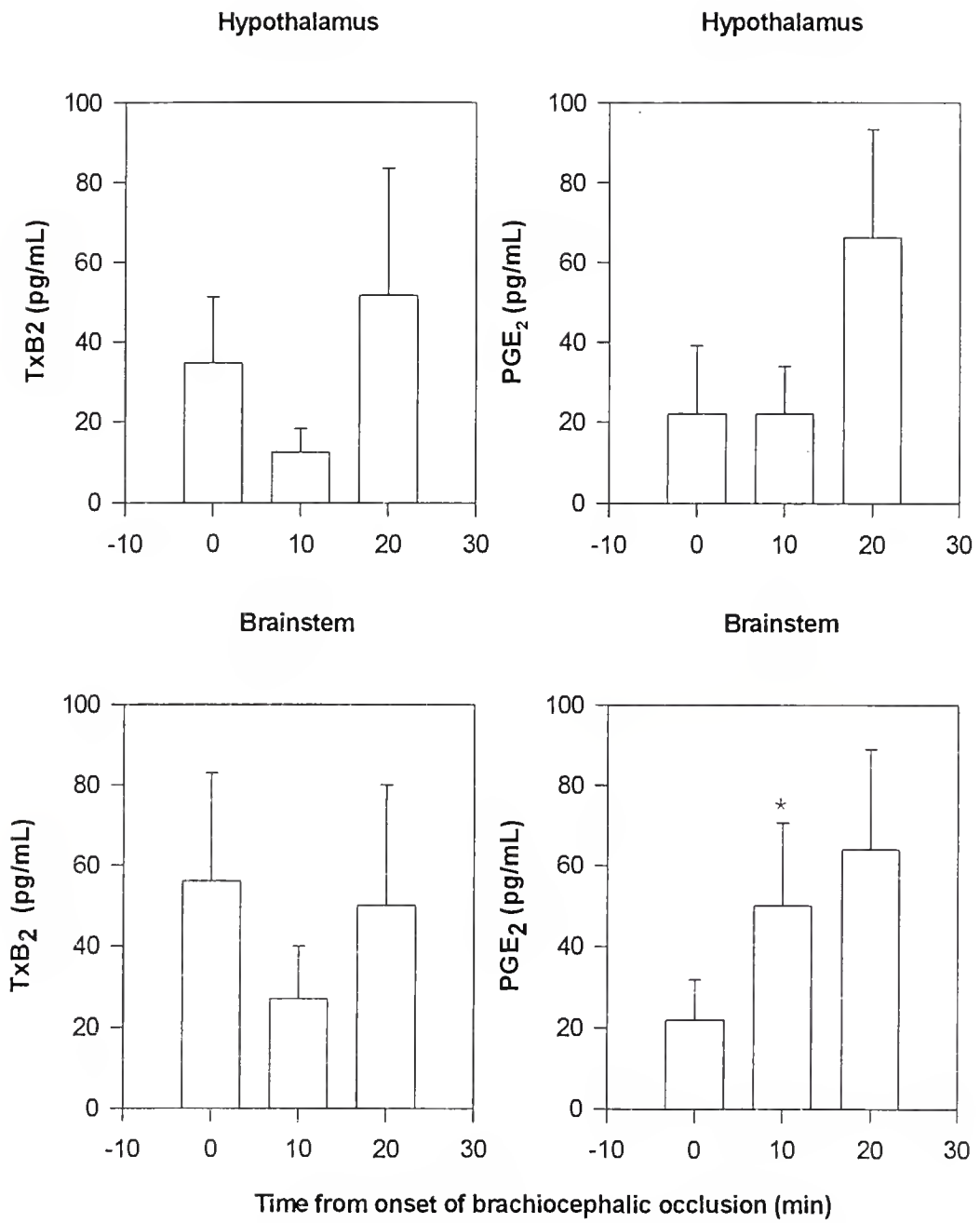



Figure 4-2. $TxB_2$ and $PGE_2$ concentrations (mean ± SE) in the hypothalamus (upper panels) and brainstem (lower panels) microdialysates from denervated fetuses before, during and 10 min after a 10-min period of brachiocephalic artery occlusion. * $p < 0.05$, indicates difference of $PGE_2$ concentration during hypotension compared to that before hypotension. N=5.

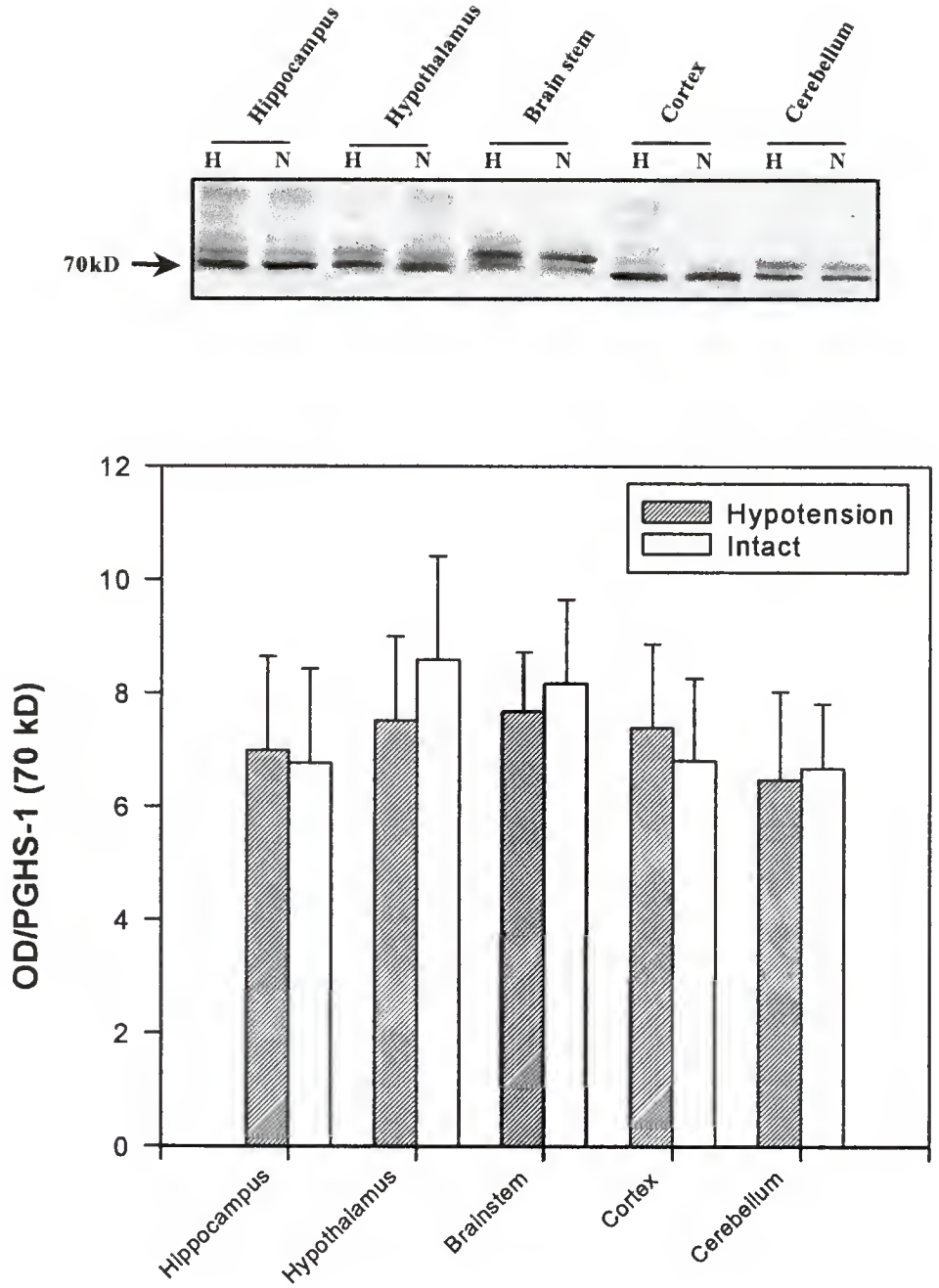



Figure 4-3. Western blot analysis of PGHS-1 protein level with PGHS-1 antibody in the intact (open bar) and denervated hypotensive (shaded bar) fetal brain tissues. Top panel: representative immunoblot showing that in each brain tissue the anti-PGHS-1 antibody recognized a 70 kDa protein band. Bottom panel: quantification of PGHS-1 protein band as optical density (OD) and values are means ± SE from five fetuses. H: represents tissue from hypotensive animal; N: represents tissue from intact animal.

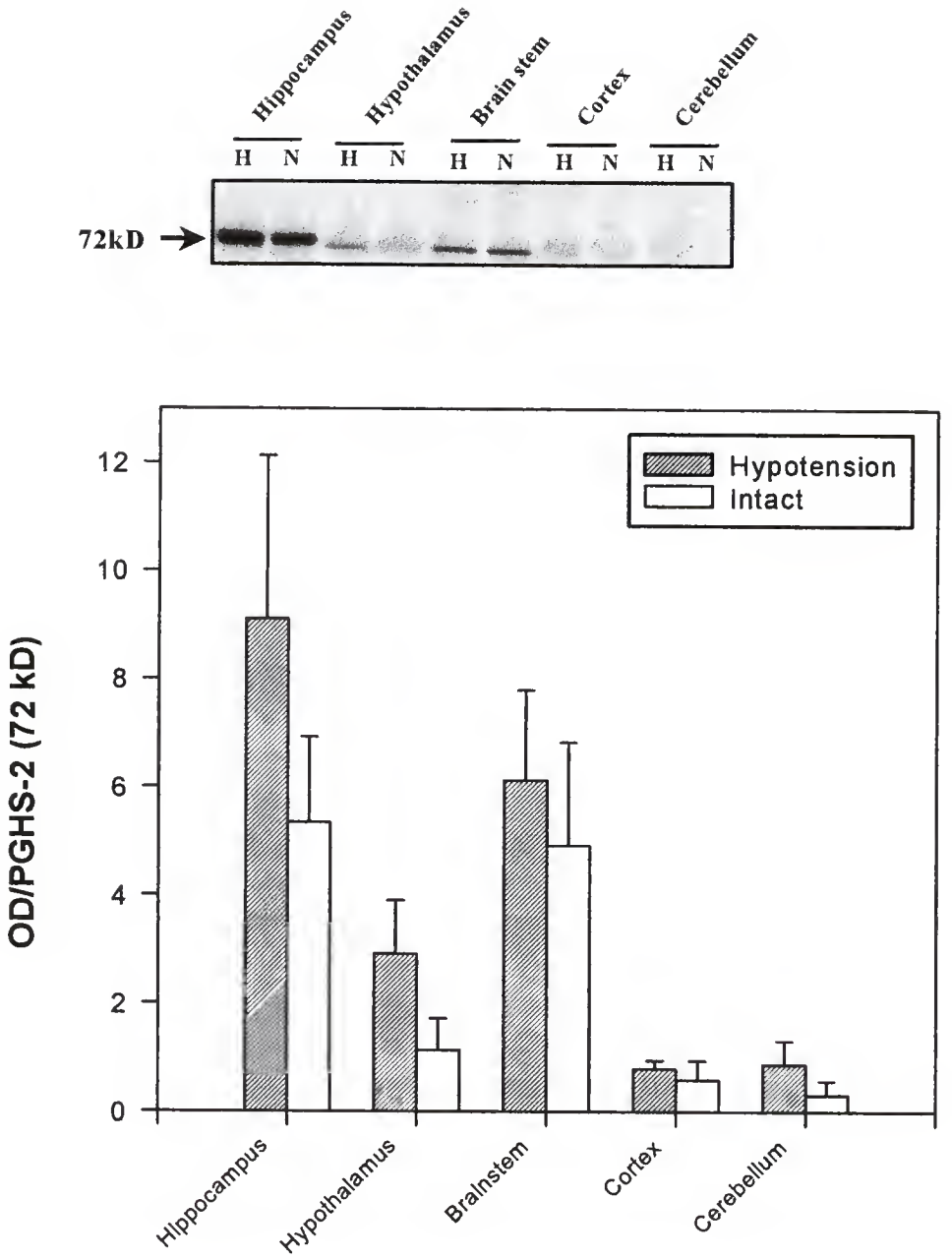
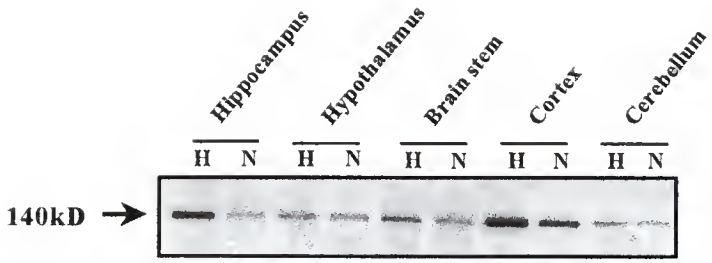



Figure 4-4. Western blot analysis of PGHS-2 protein level with PGHS-2 antibody in the intact (open bar) and denervated hypotensive (shaded bar) fetal brain tissues. Top panel: representative immunoblot showing that in each brain tissue the anti-PGHS-2 antibody recognized a single band of 72 kDa protein. Bottom panel: quantification of PGHS-2 protein bands as optical density (OD) and values are means ± SE from five fetuses. H: represents tissue from hypotensive animal; N: represents tissue from intact animal.



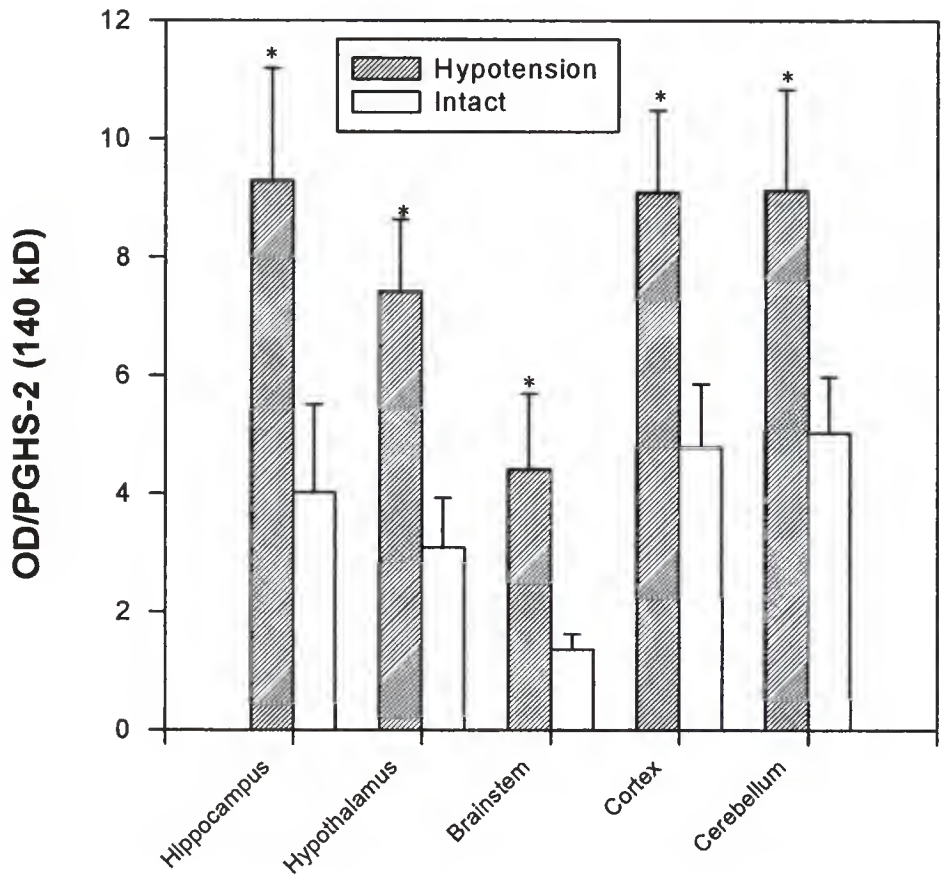




Figure 4-5. Western blot analysis of PGHS-2 protein level with PGHS-2 antibody in the intact (open bar) and denervated hypotensive (shaded bar) fetal brain tissues. Top panel: representative immunoblot showing that in each brain tissue the anti-PGHS-2 antibody recognized a single band of 140 kDa protein. Bottom panel: quantification of PGHS-2 protein as optical density (OD) and values are means ± SE from five fetuses. H: represents tissue from hypotensive animal; N: represents tissue from intact animal. *$p < 0.05$ indicates difference between groups.

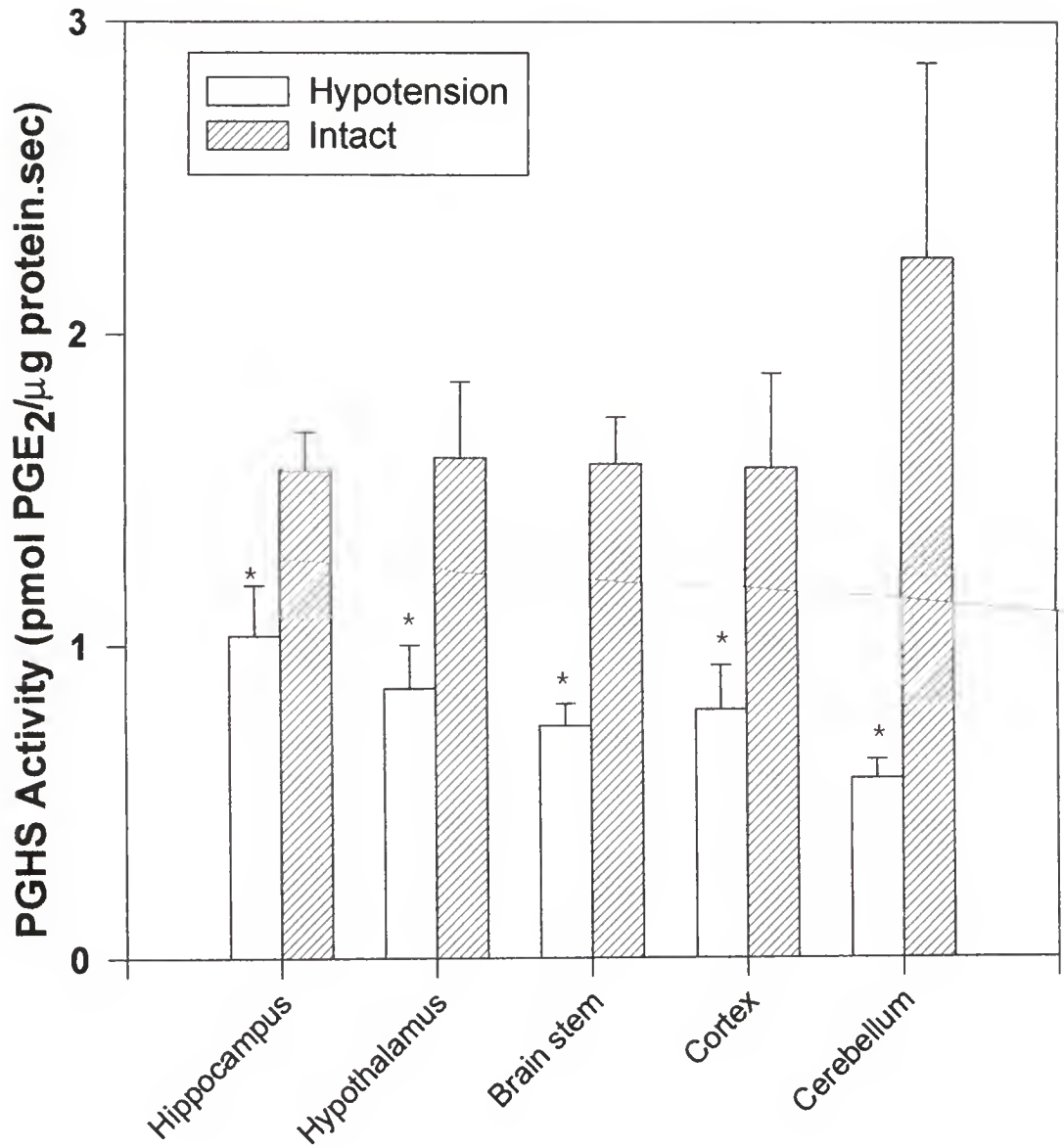



Figure 4-6. PGHS activity in the intact (shaded bar) and denervated hypotensive (open bar) fetal brain tissues. *$p < 0.05$, indicates difference between groups. Values are means ± SE from five fetuses.

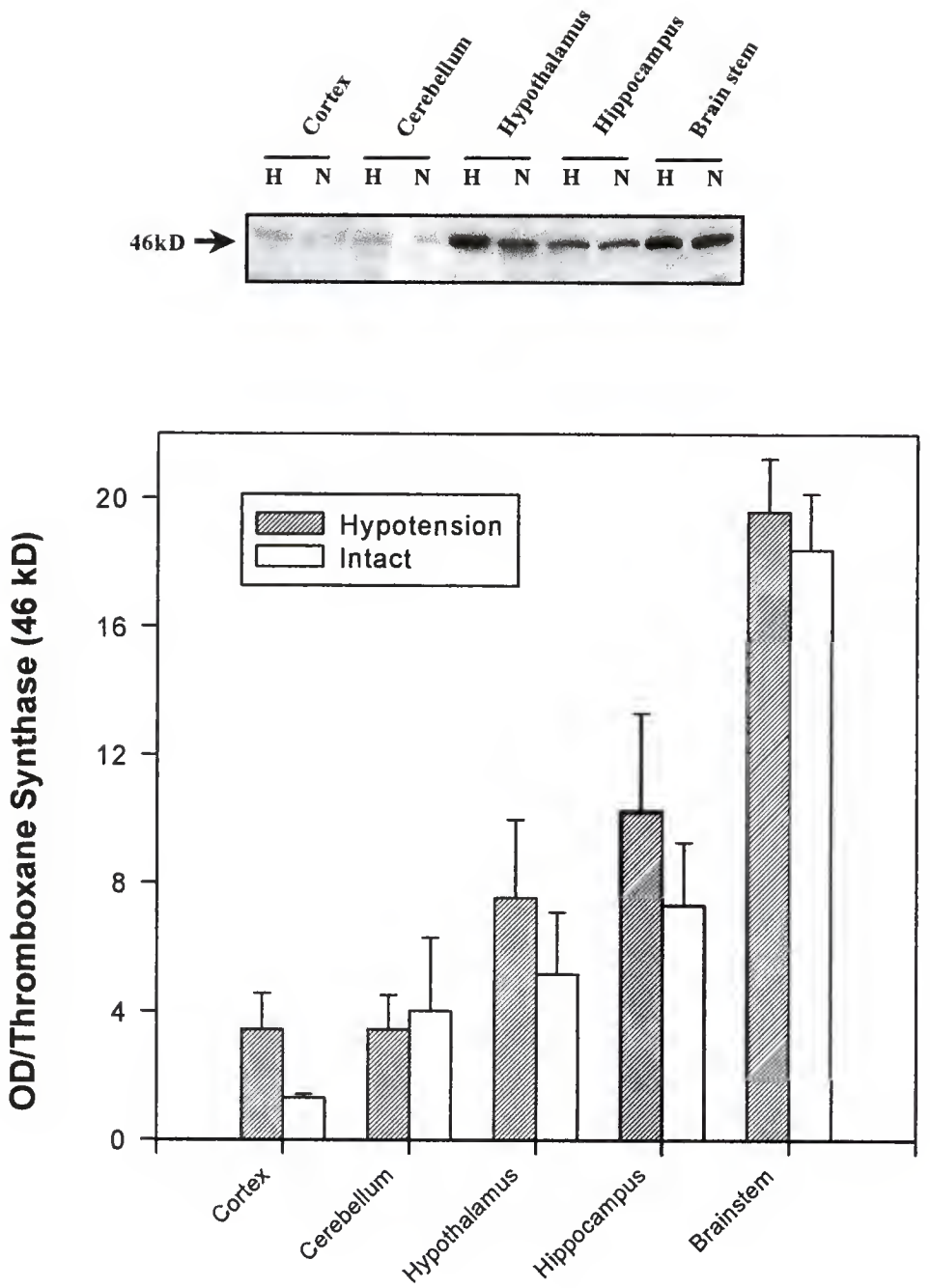



Figure 4-7. Western blot analysis of thromboxane synthase protein level with thromboxane synthase antibody in the intact (open bar) and denervated hypotensive (shaded bar) fetal brain tissues. Top panel: representative immunoblot showing that in each brain tissue the thromboxane synthase antibody recognized a 46 kDa protein band. Bottom panel: quantification of thromboxane synthase protein bands as optical density (OD). Values are means ± SE from five fetuses. H: represents tissue from hypotensive animal; N: represents tissue from intact animal.

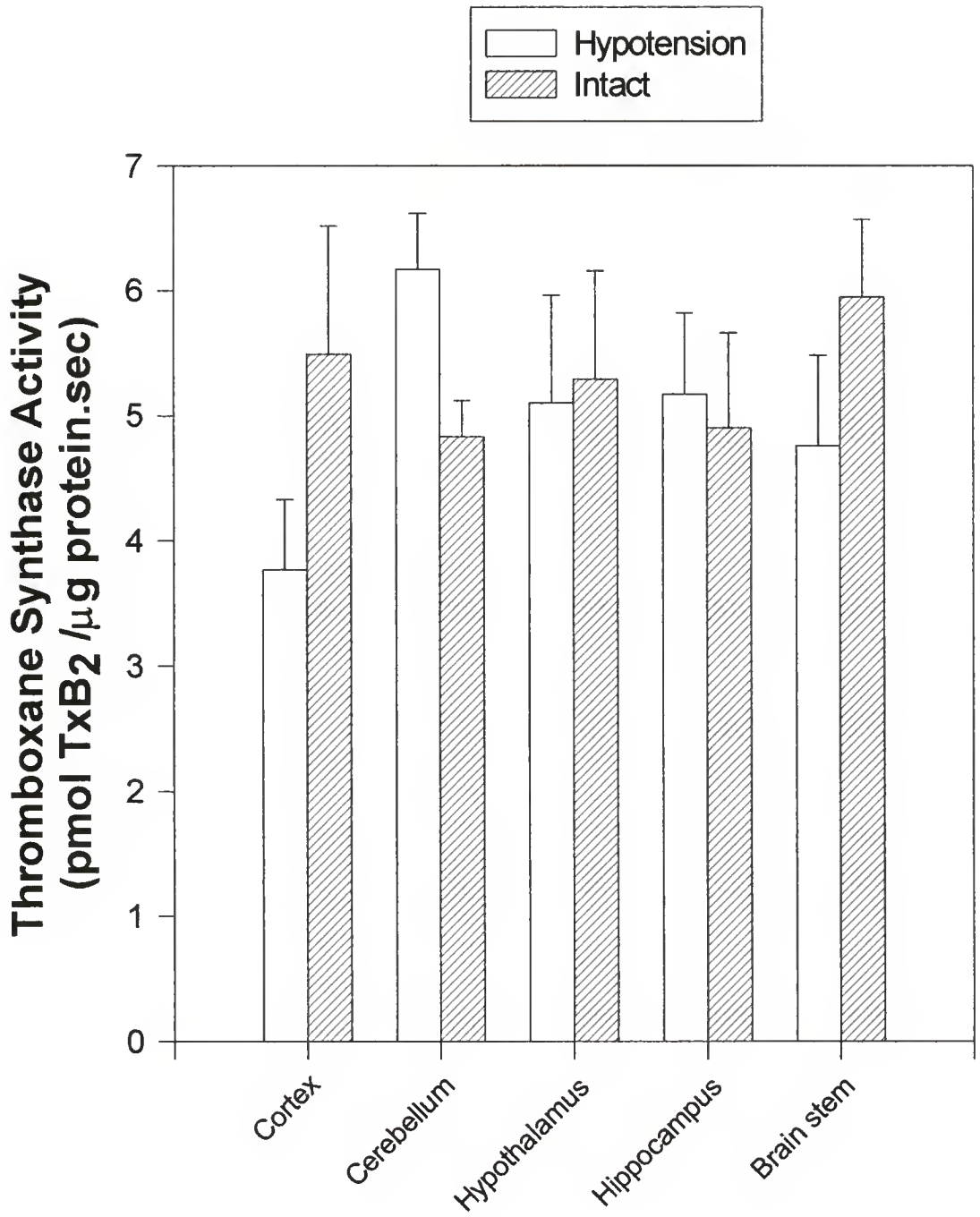



Figure 4-8. Thromboxane synthase activity in the intact (shaded bar) and denervated hypotensive (open bar) fetal brain tissues. Values are mean ± SE. N=5.

# CHAPTER 5
# EFFECT OF PROSTANOIDS AND INDOMETHACIN ON CEREBRAL BLOOD FLOW IN RESPONSE TO CEREBRAL ISCHEMIA IN LATE-GESTATION FETAL SHEEP

## Introduction

Substantial physiological data *in vivo* point to a significant role for dilator prostanoids in the regulation of cerebral vascular tone and cerebral blood flow in newborns (Leffler et al. 1993). The physiological effects of arachidonate metabolites often have opposing actions. For example, $PGE_2$ and $PGI_2$ are potent vasodilators, while $TxA_2$ and $PGF_{2\alpha}$ are vasoconstrictors (Moncada and Vane, 1978; Moskowitz and Coughlin, 1981). Arachidonic acid is liberated from damaged cell membranes during ischemia and is the source of vasoactive prostanoids which are believed to adversely influence cerebral ischemia.

Inhibition of cyclooxygenase, an enzymatic step in arachidonic acid metabolism, has an improvement effect in the outcome during cerebral ischemia. Free indomethacin crosses the blood brain barrier (Bannwarth et al. 1990). It is generally assumed that the duration of action of indomethacin is long, but the plasma half-life is relative short and the CSF concentration is closely related to that of plasma (Bannwarth et al. 1990). Indomethacin, a cyclooxygenase inhibitor, has a potential ability to prevent or attenuate the development of intraventricular hemorrhage (Ment et al. 1994). A pharmacological

dose of indomethacin is associated with a selective hypoperfusion to certain brain regions during normoxia and this result is associated with changes in the prostanoid system (Coyle et al. 1995). Indomethacin has been reported to have beneficial prophylatic effects in prevention and/or reduction of severity of intraventricular/periventricular hemorrhage in premature babies (Ment et al. 1985b; Hanigan et al. 1988; Bada et al. 1989). High dose indomethacin (5mg/kg) had been found to decrease regional and total cerebral blood flow for 120 min after administration in newborn pig (Pourcyrous et al. 1994). In piglets, indomethacin decreases CBF at rest (Leffler et al. 1985). These decreases in CBF occur concomitantly with decreases in CSF dilator PG levels (Leffler et al. 1989).

No information exists with regard to the effect of prostanoids and indomethacin on cerebral ischemia in fetuses. Our previous studies showed that prostaglandin $E_2$ concentrations increased in the interstitial fluid of the brain stem and hypothalamus, but thromboxane $B_2$ ($TxB_2$) decreased in the brain stem and hypothalamus during cerebral hypoperfusion. $TxB_2$ increased in sagittal sinus blood compared to arterial blood (Tong and Wood, 1998b). In the present study, we hypothesized that during cerebral hypoperfusion, prostanoids produced in the microvasculature and in neurons might cause vasoconstriction and reduce cerebral blood flow, and that pretreatment with indomethacin may attenuate this reduction of cerebral blood flow. To test these hypotheses, we measured regional brain blood flow in fetal sheep subjected to a 10-min of cerebral ischemia with and without pretreatment with indomethacin.

## Materials and Methods

Eight chronically catheterized fetal sheep between 126 and 136 days' gestation were used in this study. The pregnant ewes were of mixed Western breeds. These experiments were approved by the University of Florida Institutional Animal Care and Use Committee and were performed in accordance with the Guiding Principles for the Care and Use of Animals of the American Physiology Society.

### Surgical Preparation

Aseptic surgery was performed at least 5 days before the start of experiments in each animal. The fetuses were chronically instrumented with tibial artery and saphenous vein catheters and amniotic fluid catheters as previously described. Fetal hindlimbs were identified and delivered through a small hysterotomy incision near the tip of one uterine horn. We introduced polyvinyl chloride catheters into the tibial artery (.050" ID; .090" OD) and saphenous vein (0.030"ID, 0.050" OD) bilaterally and advanced the tips to the abdominal aorta and inferior vena cava, respectively. After closing the skin incisions in the fetal hindlimb, amniotic fluid catheters were sutured to the fetal skin and the hindlimb returned to the amniotic space. After placement of these catheters and closure of the hysterotomy, the fetal head was located, the uterus was incised, and the head was delivered. After a single midline incision in the skin of the neck was made, lingual arteries were identified, ligated, and catheterized with polyvinyl chloride catheters (0.050 in. OD, 0.030 in ID), with the catheter tips advanced retrograde to the lumen of the common

carotid arteries. As previously described, this catheterization technique allows measurement of common carotid arterial pressure without interruption of carotid arterial blood flow (Wood et al. 1993). The carotid sinus denervation was performed using methods described by Wood (1989). The carotid sinus nerves were identified bilaterally and cut. The walls of the common carotid arteries in this area, as well as the lingual arteries and common carotid arteries extending 0.5-1 cm rostral from the lingual-carotid arterial junction were stripped of all visible nerve fibers. After performing these denervations in the fetal neck, the fetal skin was closed. An extravascular balloon occluder (size 10) (In Vivo Metric, Healdsburg, CA) was placed around the brachiocephalic artery using a separate incision through the fourth intercostal space on the left side of chest. Then the head was returned to the amniotic cavity, and the uterus was closed. All catheters exited via a small incision in the flank of the ewes.

Ampicillin trihydrate (Polyflex; Aveco Co, Fort Dodge, IA) 500 mg was administered to the fetus via the amniotic fluid and to the mother (500-750 mg) intramuscularly at the time of surgery and again each time the fetus was studied or the catheters were flushed. Ampicillin (500-750 mg) was administered to the mother intramuscularly twice daily for five days after the surgery. All catheters were flushed and reheparinized at least once every 3 days.

Experimental Protocol

Sheep were transported to the procedure room from their pens within the Health Center Animal Resources Department at least 1 hour before the start of each experiment.

Each fetus was studied twice. Experiments consisted of a 90 min pre-occlusion control period (-90 to 0 min), a 10 min occlusion period (0 to 10 min) and a 10 min post-occlusion recovery period (10 to 20 min). The occlusion of brachiocephalic artery was complete. In one experiment on each fetus, the vehicle of indomethacin, 0.1 M phosphate buffer (PB) was injected intravenously, and in the other experiment 0.2 mg/kg indomethacin (an inhibitor of cyclooxygenase) was injected intravenously 90 min prior to the 10 min period of hypotension. PB and indomethacin were randomly administrated to the fetal sheep. In each experiment, the extravascular occluder was inflated for 10 min to produce arterial hypotension. Fetal arterial blood samples were drawn from tibial artery catheters (descending aorta) at 90 and 10 min before the start of the period of occlusion, at the end of the 10 min period of occlusion, and 20 min after the onset of occlusion. One ml arterial blood sample was drawn before the experiment to measure blood gases. Blood samples (3 ml) were collected into chilled polystyrene tubes containing 150 µl of 0.5 M EDTA. Separate blood samples (1 ml) were collected into chilled polypropylene tubes containing 50 µl 0.5 M EDTA and 40 µg/ml indomethacin for measuring $TxB_2$ and $PGE_2$. Tubes were kept on ice until the end of the experiment and then centrifuged for 20 min at 3000 x *g* at 4 ° C. Plasma was separated and stored in separate aliquots at -20 ° C.

Plasma adrenocorticotropic hormone (ACTH), cortisol, and arginine vasopressin (AVP) concentrations were measured by specific radioimmunoassay. These assays have been described in detail in chapter 3 and (Wood et al. 1993; Raff et al. 1991). $PGE_2$ and thromboxane $B_2$ ($TxB_2$, a stable metabolite of $TxA_2$) were measured using enzyme-linked immunoassay kits purchased from Cayman Chemical. Before assay, the prostanoids were

extracted from acidified plasma with 6 volumes of ethyl acetate. The recovery using this protocol averages approximately 60%, and the extracted prostanoids dilute parallel to the standard curves. This assay has been described in chapter 3.

Fetal arterial blood pressure and amniotic fluid pressure values were measured continuously during the 110 min experiments using a Grass recorder and Statham P23 ID pressure transducers. These hemodynamic variables were sampled, and analog-to-digital conversions were performed at 2-s intervals using an IBM PC computer. The data collection was achieved using ASYSTANT$^{+}$ software (Asyst Technologies, Rochester, NY). All fetal intravascular pressure were corrected by subtraction of amniotic fluid pressure.

Immediately before and 5 minutes after the onset of hypotension, colored microspheres (15 μm, Dye-Track, Triton Technologies, San Diego, CA) were injected through the venous catheter and simultaneous reference blood samples were drawn from the lingual arterial catheters at the rate of 3 ml/min for 1 min 30 sec. Four colors of microspheres were used in each animal for measuring cerebral blood flow. These techniques have been described in detail (Lakhdia et al. 1998).

At the end of the second experiment, the pregnant ewes (and the fetus) were euthanized with an overdose of pentobarbital. The fetal brains were dissected for microsphere extraction.

Estimation of Cerebral Blood Flow Using Colored Microspheres

The fetal brains were dissected into cerebral cortex, brain stem, hippocampus, hypothalamus, cerebellum, and pituitary. Same amount of tissues were used in this study.

The dissected tissues were dissolved in 4 ml of 4 M potassium hydroxide and 0.2% Tween 80 for 48 hours and filtered through an 8 μm thickness polyester membrane filter. The membrane (containing the microspheres) was placed in a microcentrifuge tube to air dry overnight. Dimethylformamide (150 μl) was added to the tube to leach the color microsphere from the membrane. Then, the tube was centrifuged and 80 μl of the supernatant was pipetted to a cuvette. The absorbance spectra (ABS) was quantified using a spectrophotometer and the appropriate software for least-squares solution of absorbance of each dye. The reference blood samples were processed similarly, except that 16 M potassium hydroxide was used.

Cerebral blood flow in each tissue was calculated by the following equation:

$$CBF = (\text{tissues ABS}/\text{reference ABS}) * \text{reference flow rate}$$

Statistical Analyses

Change in mean arterial blood pressure over time was analyzed by one-way ANOVA. Changes in the values of fetal hormonal and prostanoid variables over time and between groups were analyzed using two-way analysis of variance (ANOVA). A multiple comparison of mean values was performed using Student's Newman-Keuls test. The hormonal data were not distributed normally. All ANOVAs performed on hormonal data were calculated after logarithmic transformation to correct heteroscedasity of the data. Fetal CBFs with PB/indomethacin treatment and occlusion were analyzed using three-way analysis of variance. A multiple range test was performed using Duncan's test. A

significance level of $P < 0.05$ was used to reject the null hypothesis in all tests. Analyses were performed using SigmaStat software (Jandel Scientific, San Rafael, CA).

## Results

### Blood Gases and Blood Pressure

The blood gases measured before phosphate buffer and indomethacin injections indicated that all fetuses were healthy during the experiments (table 5-1). Injection of indomethacin did not alter fetal blood gases. Fetal mean arterial blood pressure before, during and after brachiocephalic occlusion were presented in Fig. 5-1. Lingual arterial blood pressure decreased significantly ($p < 0.001$) by the brachiocephalic occlusion in both PB and indomethacin treated groups. In the PB group, mean lingual arterial blood pressure decreased from $41.9 \pm 3.1$ to $16.5 \pm 3.3$ mm Hg (mean $\pm$ SE). In the indomethacin group, blood pressure decreased from $41.6 \pm 2.5$ to $14.6 \pm 3.0$ mm Hg. Blood pressure returned to baseline level after the occluder was released. Femoral arterial blood pressure increased significantly ($p < 0.05$) by the brachiocephalic occlusion in PB-treated group, from $42.9 \pm 3.7$ to $64.9 \pm 7.0$ mm Hg. In the indomethacin-treated group, mean femoral arterial blood pressure during occlusion ($51.1 \pm 4.2$ mm Hg) was higher ($P < 0.05$) than that after occlusion ($39.3 \pm 1.7$ mm Hg). These data suggested that the blood flow shunted to peripheral tissues when the cerebral perfusion pressure was reduced.

Plasma Prostanoid Concentrations

The mean values of fetal plasma prostaglandin $E_2$ and thromboxane $B_2$ concentrations are summarized in Table 5-2. There was no difference in the initial values of prostanoid concentrations before PB and indomethacin injections. After indomethacin injection, plasma $PGE_2$ and $TxB_2$ concentrations decreased significantly ($p < 0.05$), indicating that indomethacin effectively inhibited cyclooxygenase activity. Compared to the preocclusion value, hypotension increased plasma $PGE_2$ and $TxB_2$ concentrations in PB-treated group, but there was no statistically significant difference.

Table 5-1. Arterial blood gases before PB and indomethacin injections in denervated fetuses

| | PB | Indomethacin |
|---|---|---|
| pH | 7.35 ± 0.01 | 7.35 ± 0.01 |
| $PaO_2$ (mm Hg) | 19.28 ± 0.91 | 20.42 ± 0.68 |
| $PaCO_2$ (mm Hg) | 51.88 ± 2.43 | 49.53 ± 1.72 |

Values are means ± SEM.

Regional Cerebral Blood Flow

The mean values of fetal regional cerebral blood flow (rCBF) are reported in table 5-3. In PB-treated group, brachiocephalic occlusion significantly decreased rCBF in cerebral cortex (from 1.42 ± 0.22 to 0.30 ± 0.09 ml/g.min) (mean ± SE), brain stem (from 2.43 ± 0.39 to 0.86 ± 0.25 ml/g.min), hippocampus (from 1.37 ± 0.18 to 0.49 ± 0.17 ml/g.min), hypothalamus (from 2.20 ± 0.33 to 1.05 ± 0.40 ml/g.min) and cerebellum (from 1.85 ± 0.27 to 0.41 ± 0.15 ml/g.min) ($P < 0.05$).

Table 5-2. Fetal plasma prostanoid concentrations in denervated fetuses

| | PB (n = 6) | Indomethacin (n = 7) |
|---|---|---|
| $PGE_2$ (-90 min) | 192.2 ± 79.8 | 181.4 ± 31.9 |
| $PGE_2$ (-10 min) | 163.2 ± 74.5 | 21.1 ± 8.5*† |
| $PGE_2$ (10 min) | 229.8 ± 119.5 | 44.3 ± 25.1* |
| $PGE_2$ (20 min) | 265.7 ± 142.4 | 17.3 ± 5.4*† |
| $TxB_2$ (-90 min) | 85.8 ± 43.4 | 92.2 ± 34.6 |
| $TxB_2$ (-10 min) | 65.6 ± 25.9 | 20.5 ± 5.6* |
| $TxB_2$ (10 min) | 118.9 ± 47.7 | 10.5 ± 3.9*† |
| $TxB_2$ (20 min) | 87.2 ± 31.2 | 22.9 ± 8.1*† |

Values are means ± SEM. Time is relative to the start of hypotension (t = 0 min). *$p < 0.01$, indicates difference due to the time difference within indomethacin group (Student-Newman-Keuls test). †$p < 0.05$, indicates difference between PB and indomethacin group (Duncan's test).

Table 5-3. Fetal cerebral blood flow of PB and indomethacin treatments

| | PB | | Indomethacin | |
|---|---|---|---|---|
| | Normotension (n = 5) | Hypotension (n = 6) | Normotension (n = 7) | Hypotension (n = 7) |
| Cortex | 1.42 ± 0.22 | 0.30 ± 0.09* | 1.22 ± 0.12 | 0.59 ± 0.11 |
| Brain stem | 2.43 ± 0.39 | 0.86 ± 0.25* | 2.21 ± 0.29 | 1.22 ± 0.28† |
| Hippocampus | 1.37 ± 0.18 | 0.49 ± 0.17* | 1.34 ± 0.13 | 0.76 ± 0.15 |
| Hypothalamus | 2.20 ± 0.33 | 1.05 ± 0.40* | 1.78 ± 0.21 | 1.06 ± 0.12 |
| Cerebellum | 1.85 ± 0.27 | 0.41 ± 0.15* | 1.64 ± 0.20 | 0.70 ± 0.19† |

Values are means ± SEM. *$p < 0.05$, indicates the difference between normotension and hypotension in the PB-treated group. †$p < 0.05$, indicates the difference between normotension and hypotension in the indomethacin group. All multiple range test procedures were done in Duncan's test.

Indomethacin pretreatment (n =7) slightly decreased but did not significantly change basal rCBFs. Hypotension decreased rCBF in the indomethacin-treated group, especially in cerebral cortex (from 1.22 ± 0.12 to 0.59 ± 0.11 ml/g.min), brain stem (from 2.21 ± 0.29 to 1.22 ± 0.28 ml/g.min) and cerebellum (from 1.64 ± 0.20 to 0.70 ± 0.19 ml/g.min) ($p < 0.05$). Regional CBFs in the indomethacin-treated group during brachiocephalic occlusion were higher than that in the PB group during occlusion. Indomethacin increased rCBF in cerebral cortex, brain stem, hippocampus, hypothalamus and cerebellum by 49%, 30%, 36%, 18% and 42% respectively during hypotension. Three-way analysis of variance (ANOVA) performed on fetal CBFs over PB/indomethacin treatment and blood pressure change, revealed that there was a significant interaction between PB/indomethacin treatment and blood pressure change ($F = 5.224$, $p < 0.05$). Duncan's test was used in the multiple range test and the results were reported in table 5-3. These data suggested that indomethacin decreased the reduction in fetal cerebral blood flow during cerebral hypoperfusion.

Plasma Hormone Concentration

Indomethacin treatment did not alter ACTH and AVP responses to hypotension. Plasma ACTH concentration was increased significantly from 74 ± 14 to 1811 ± 1009 pg/ml (mean ± SE) in the fetuses treated with PB, and from 200 ± 52 to 1490 ± 482 pg/ml treated with indomethacin by the brachiocephalic occlusion. Plasma AVP concentration was increased significantly from 11 ± 2 to 47 ± 18 pg/ml in the fetuses treated with PB, and from 20 ± 6 to 74 ± 32 pg/ml for those treated with indomethacin by the

brachiocephalic occlusion. Plasma cortisol concentration was increased from 11 ± 2 to 21 ± 5 ng/ml in the fetuses treated with PB, and from 16 ± 4 to 28 ± 8 ng/ml treated with indomethacin by the hypotension. There was no significantly difference between PB and indomethacin treatment (analyzed by two-way ANOVA) (Fig. 5-2). This is consistent with our previous studies that indomethacin did not further attenuate hormonal responses to hypotension in denervated fetuses (Tong et al. 1998a).

## Discussion

The present study was designed to test the roles of prostanoids and indomethacin in the control of fetal cerebral blood flow (CBF) during cerebral ischemia. It has been observed that prostanoids are important in modulating perinatal cerebral hemodynamics in human beings and some animals (Leffler and Busija, 1987b). Our previous study demonstrated that $TxB_2$ secretions increased significantly in sagittal sinus blood during cerebral hypoperfusion, and $PGE_2$ concentrations increased significantly in fetal brain stem and hypothalamus interstitial fluids. We tested the hypothesis that indomethacin may compromise fetal CBF by inhibiting these prostaglandins productions in the brain during cerebral ischemia.

Papile et al. (1985) demonstrated that CBF autoregulation was developed in the preterm fetal lamb and the range of autoregulation was narrowed in the preterm lamb. It was speculated that the fetal brain under normal physiological conditions in utero may not protect against acute fluctuations in blood pressure, particularly hypotension (Papile et al. 1985).

In this study, brachiocephalic occlusion decreased the same degree of lingual arterial blood pressure which perfuses brain in both PB and indomethacin-treated fetuses. Also, hypotension decreased cerebral blood flow in both groups. Interestingly, this study showed that indomethacin increased fetal regional CBF by 30-49% compared to PB-treated group when blood pressure was reduced by about 60% (below 20 mm Hg). We believe that the increases in CBF in indomethacin pretreatment were due to inhibiting constrictor prostaglandin by indomethacin. Indeed, $TxB_2$ concentration decreased significantly after indomethacin injection in this study. Indomethacin is highly lipid soluble but also is highly protein-bound; it crosses the intact meninges by simple diffusion (Bannwarth et al. 1990). Indomethacin can be found in the brain within 30 min of systemic administration (Bannwarth et al. 1990). In contrast to our findings, Leffler et al. (1989) found that indomethacin decreased CBF in newborn animals by inhibiting dilator prostaglandins. Indomethacin possesses several actions other than cyclooxygenase inhibition (Burch et al. 1983; Jugdutt et al. 1979) that may account for the CBF reduction during a decrease in blood pressure within the range of CBF autoregulation in newborns (Leffler et al. 1986). Chemtob et al. (1990a) demonstrated that in newborn piglets, inhibition of $TxB_2$ synthesis was associated with an extension of the lower blood pressure limit of CBF autoregulation and an attenuation in the decrease in CBF below this blood pressure. These studies suggested that there were differences in the cerebrovascular action of indomethacin in different degree of hypotension.

In this experiment design, all fetuses were carotid sinus denervated in order to be consistent with the previous study (Tong et al. 1998a) and this may not affect cerebral

circulation in response to ischemia. The fetal regional cerebral blood flow was in the normal range in PB-treated animals. Itskovitz and Rudolph (1982) demonstrated that sinoaortic denervation of the preterm fetal lamb dose not alter resting heart rate or blood pressure and has no effect on CBF within the autoregulation range (75-80 mm Hg). It appeared that the autonomic nervous system does not appear to have a major role in the control of cerebral circulation (Traystman, 1981).

The hormonal responses to cerebral hypoperfusion of this study were consistent with our previous experiments designed to test the endogenous prostanoids modulating the hormonal responses to arterial hypotension and the effects of denervation on these responses (Tong et al. 1998a). Carotid sinus denervation attenuated ACTH and AVP responses to hypotension and endogenous prostanoids did not further affect these hormonal responses in late-gestation fetal sheep.

In conclusion, this study demonstrated that indomethacin had beneficial effect on fetal regional cerebral blood flow in response to cerebral ischemia in late-gestation fetal sheep. This effect was due to inhibiting constrictor prostaglandin synthesis in the brain by indomethacin. This may be an alternative way of indomethacin to treat severe cerebral ischemia in fetuses.

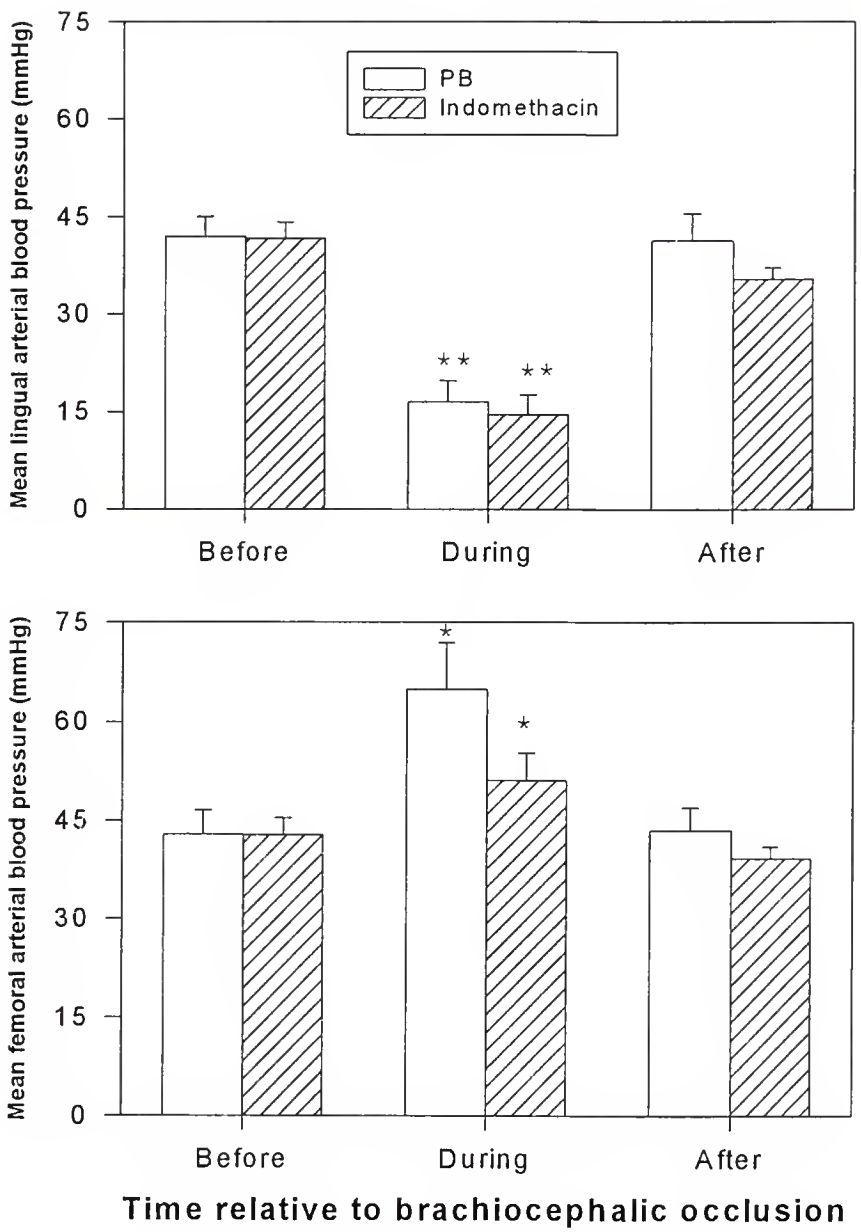



Figure 5-1. Fetal mean lingual (top panel) and femoral (bottom panel) arterial blood pressure (MAP) before, during and after a 10-min period of brachiocephalic occlusion (BCO) in the denervated fetuses. There were no significant differences in MAP between PB and indomethacin treated groups. Mean values are accompanied by vertical bars representing 1 SE. **$p < 0.01$ and *$p < 0.05$, compared to baseline MAP by BCO.

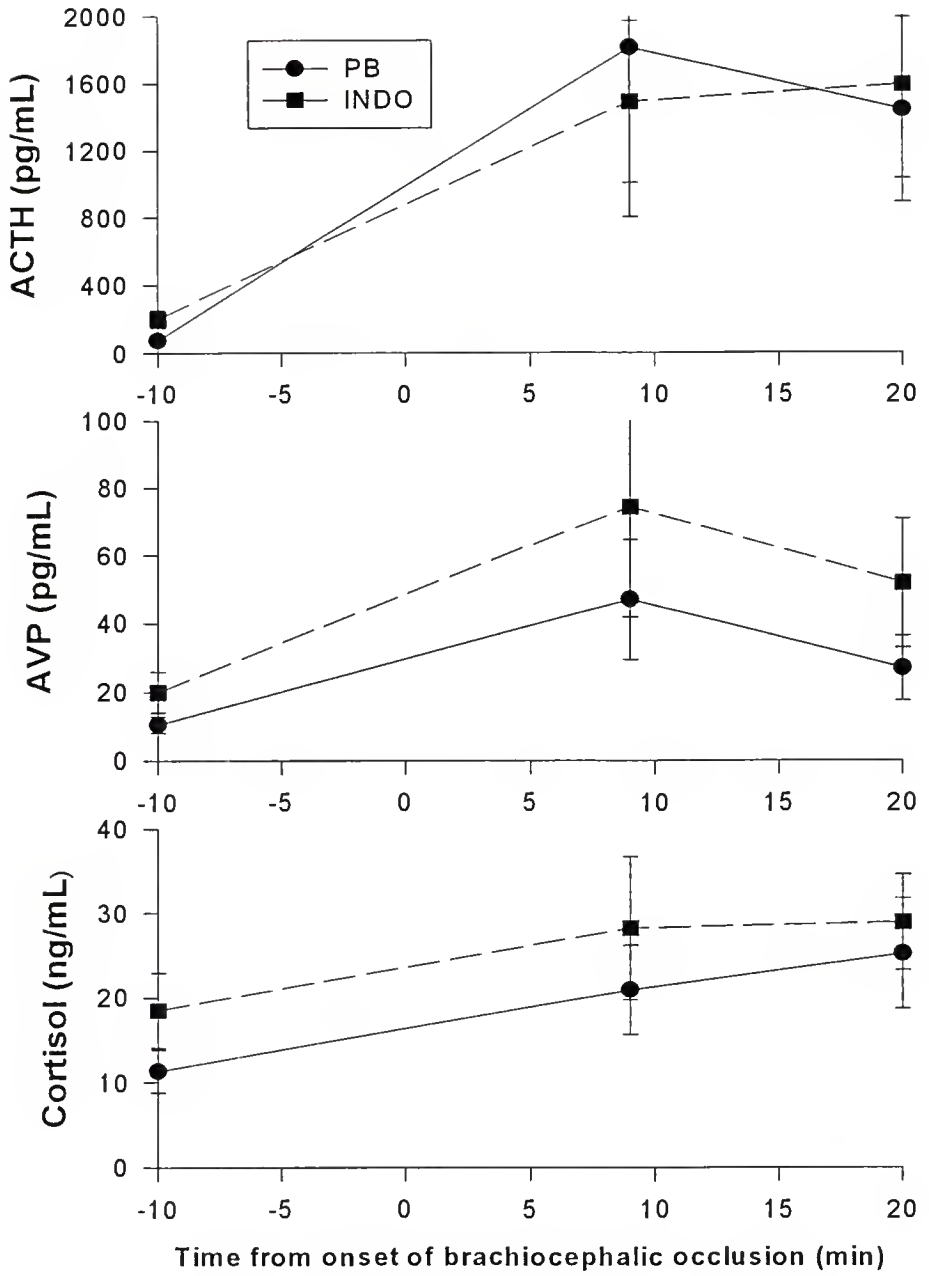



Figure 5-2. Fetal plasma ACTH, AVP and cortisol concentrations before, during and after a 10-min period of brachiocephalic occlusion (BCO) in the denervated fetuses. Mean values of hormones in phosphate buffer (●, solid lines) and indomethacin (■, dash lines) treated fetuses are accompanied by vertical bars representing 1 SE. Two-way ANOVA did not show significant effect between groups.

# CHAPTER 6
# EXPRESSION OF PGHS-2 AND FOS GENE INDUCED BY CEREBRAL ISCHEMIA IN FETAL SHEEP BRAIN

## Introduction

Prostaglandin endoperoxide synthase (PGHS) is a rate-limiting enzyme in prostaglandin and thromboxane syntheses. There are two isoforms of prostaglandin H synthase (PGHS), inducible PGHS-2 encoded by a 4 kilobase mRNA, and constitutive PGHS-1 encoded by a 2.8 kilobase mRNA. Promoter analysis suggests that PGHS-1 is a member of the housekeeping gene family (Wang et al. 1993) and PGHS-2 is a member of immediate early gene (IEG) family (Xie et al. 1993). The promoter sequence of PGHS-2 gene contained a TATA box and a variety of enhancer elements, including an activator protein-1 (AP-1) site (Xie et al. 1993). The PGHS-2 gene may be transcriptionally regulated at its AP-1 binding element by transcription factor c-fos (Chiu et al. 1988; Xie et al. 1993); however, promoter analysis of the PGHS-1 gene reveals no putative AP-1 domain (Wang et al. 1993).

There are several families of the IEGs. They include the c-fos and c-jun that encode transcription factors. Products of the fos family bind to members of the jun family and form AP-1 transcription factor in the promoter of target genes, which in turn regulate the expression of late response genes that produce long-term changes in cells (Chiu et al.

1988). Cerebral ischemia induced proto-oncogene expression in animal models (Kindy et al. 1991; Onodera et al. 1989). It is suggested that the expression of c-fos after ischemia may be immediately activated through NMDA receptors and may spread to surrounding regions in a manner sensitive to reductions in rCBF (Shimazu et al. 1994).

Immunocytochemistry studies showed that PGHS are located intracellularly and that they function to produce prostaglandins for use as intracellular second messengers or as paracrine mediators (Breder et al. 1992). PGHS is normally expressed throughout the brain in discrete populations of neurons (Breder et al. 1995) and detected in several non-neuronal cells such as macrophages (Lee et al. 1992), astrocytes (O'Banion et al. 1996), and endothelial cells (Habib et al. 1993). PGHS-2 was seen in many neurons that were also immunoreactive for Fos in the brain (Holtz et al. 1996). Brain ischemia was reported to induce PGHS-2 mRNA and protein (Collaco-Moraes et al. 1996). However, Fos was not involved in the expression of PGHS-1 mRNA during cerebral ischemia (Holtz et al. 1996). PGHS-2 induction in neurons at risk to die could contribute to neuronal death in brain ischemia, this is supported by our previous study that the cyclooxygenase inhibitor, indomethacin, reduced brain damage in global ischemia (Tong and Wood, unpublished data).

Our previous studies demonstrated that cerebral ischemia induced PGHS-2 but not PGHS-1 protein expression in brain tissues in late-gestation fetal sheep. We also found that prostanoids are released into cerebrovasculature and brain interstitial fluids in response to the cerebral ischemia in fetal sheep. One explanation for the rise in prostaglandin levels in the brain during the cerebral ischemia may be the increased

transcription of the PGHS-2 gene and the translational product. We also hypothesized that the transcription of the PGHS-2 gene in the ischemic brain would be affected by protein translated from c-fos mRNA, due to its AP-1 binding domain. We tested these hypotheses by measuring levels of PGHS-2 mRNA and protein and Fos, and localizing their expressions in the fetal brain during cerebral ischemia.

## Materials and Methods

### Materials

Polyclonal rabbit antibodies specific to PGHS-1 and PGHS-2 were purchased from Oxford Biomedical Research (Oxford, MI). Polyclonal rabbit antibody specific to thromboxane synthase was purchased from Cayman Chemical (Ann Arbor, MI). Polyclonal rabbit antibody specific to Fos was purchased from Oncogene Science (Cambridge, MA). Stable DAB was purchased from Research Genetics (Huntsville, AL). ImmunoPure ABC staining kit was purchased from Pierce (Rockford, IL). Oligo(dT)15 primer, *Taq* DNA polymerase, DNA ladder, RNAase inhibitor, dNTP were from Promega (Madison, WI). SuperScript M-MLV reverse transcriptase was purchased from GIBCO-BRL (Gaithersburg, MD). Guanidine thiocyanate, MOPS, Tris, SDS, EDTA, chloroform, isopropanol were from Sigma (St. Louis, MO). All other chemicals were from Fisher.

### Animals

The pregnant ewes were of mixed Western and Florida native breeds were obtained from IFAS at University of Florida. The fetal sheep between 126 and 136 days'

of gestation age were used in this study. These experiments were approved by the University of Florida Institutional Animals Care and Use Committee and were performed in accordance with the Guiding Principles for the Care and Use of Animals of the American Physiology Society.

Tissue Preparations

Animals for immunocytochemistry study

There were three groups in this study: one group of fetuses (n = 3) were intact for control; carotid sinus denervation was performed in another group of fetuses, they were subjected to a 10-min period of cerebral hypoperfusion (reduced blood pressure approximately 60%) and were sacrificed at 30 min (n=4) and 2 hr (n=3) after the onset of hypotension. An extravascular balloon was put around the brachiocephalic artery after the left chest was opened to produce cerebral hypoperfusion. All fetal brains were perfused with heparinized phosphate-buffer saline followed by 4% paraformaldehyde (PFA). The whole brain (including pituitary) was removed and immersed fixed in 4% PFA for a further 24 h at 4°C. Then it was immersed in 70% ethanol with changes every 1-2 days until processed. The brain was cut into cerebral cortex, cerebellum, hypothalamus, hippocampus and brain stem in coronal sections and dehydrated with increasing concentration of ethanol and xylene, and embedded in paraffin. 10 $\mu$m thick tissue sections were cut and affixed on poly-L-lysine coated slides, incubated at 42°C for 2 h, then stored at -20°C until processed.

Animals for the expression of mRNA study

Intact and carotid sinus denervated fetuses (4 each group) were subjected to a 10-min period of cerebral hypoperfusion (reduced blood pressure approximately 60%) and were sacrificed 30 min after the onset of hypotension. The fetal brain was quickly removed and dissected rapidly into cerebral cortex, cerebellum, hypothalamus, hippocampus, brain stem and pituitary, snap frozen immediately in a dry ice and acetone bath, then stored at -80°C.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR) Quantification of mRNAs

The levels of PGHS mRNA in fetal brain tissues were determined by RT-PCR. Total cellular RNA was extracted by a guanidine thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Fetal brain tissues (100 mg) were homogenized in 2 ml of 4 M guanidine thiocyanate denaturing solution, then extracted with phenol-chloroform. The RNA was precipitated from aqueous phase by the addition of isopropanol (3 ml) and ethanol and pelleted by centrifugation at 10,000 g for 20 min. The quantity of total RNA was measured by spectrophotometer and its integrity was assessed by 1% non-denaturing agarose gel electrophoresis and visualization of the 18s and 28s ribosomal RNA bands with ethidium bromide staining.

Total RNA (2 $\mu$g) was reversed transcribed to cDNA in 1 X buffer containing 200 U of superScript II reverse transcriptase, 0.5 mM dNTP, 0.01 M dithiothreitol, 40 U RNase inhibitor and 0.5 $\mu$g of oligo$(dT)_{15}$ primers in a total volume of 20 $\mu$l according to

the manufacturer's protocol (GIBCO). Briefly, the mixture was incubated for 50 min at 42°C, and the reaction was terminated by heating at 75°C for 15 min. The cDNA sample (3 $\mu$l) was amplified with specific primers for PGHS-1, PGHS-2 and β-actin. PCR reactions were carried out in 1 X PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 $\mu$M primers and 2 U of *Taq* DNA polymerase. The cycling program for PGHS-1 is 94°C for 1 min for denaturation, 56°C for 1 min for annealing and 72°C for 1 min for elongation for 33 cycles; for PGHS-2 is 94°C for 1 min, 61°C for 1 min and 72°C for 1 min for 30 cycles; for β-actin is 94°C for 1 min, 56°C for 1 min and 72°C for 1 min for 30 cycles. The PCR reaction products were separated by electrophoresis on 2% agarose gels. The gels were stained with ethidium bromide and visualized under UV transillumination. The density of PCR bands were analyzed using a densitometer and image analysis software (Bio-Rad, Hercules, CA). The yield of the amplified product was tested to be linear for amount of input RNA and PCR cycle number. For RT-PCR, the following optimized conditions were chosen: 3 $\mu$l RT reaction/33 Cycles for PGHS-1 mRNA amplification, 3 $\mu$l RT reaction/30 Cycles for PGHS-2 and 3 $\mu$l RT reaction/30 Cycles for β-actin.

PCR primers were selected from the published sheep cDNA sequences (Zhang et al. 1996; DeWitt and Smith, 1988) with the aid of the PRIMER DESIGN software and synthesized in ICBR at University of Florida. The expression of the PGHS-1 gene was determined by amplification of a 618-bp region of the PGHS-1 cDNA sequence (245-863). Using the sense upstream primer sequence 5'- CTTAGCCGCTACCAATGT-3' and the antisense downstream primer sequence 5'-GAGCATCTGGTACTTCAGCTTC-3'. For PGHS-2, expression of the gene was determined by amplification of a 881-bp region

of the PGHS-1 cDNA sequence (1395-2276). With the sense upstream primer sequence 5'-TGACCAGAGCAGAGAGATGA-3' and the antisense downstream primer sequence 5'-AGAAGGACCAGTGAGTAGACAG-3'. As a positive control for each RNA preparation, a rat β-actin sequence was also amplified simultaneously in adjacent tubes. The sense upstream primer of rat β-actin cDNA sequence 5'-GAGAAGATGACCCAGATCATGT-3' and the antisense downstream primer sequence 5'-ACTCCATGCCCAGGAAGGAAG-3'. All other conditions were identical to those for PGHS cDNA amplification.

The semi quantitative RT-PCR methodology was used to evaluate mRNAs for PGHS-1 and PGHS-2. The band intensity of the specific cDNA was compared with the band intensity of β-actin and the result was expressed as ratio to β-actin.

Immunocytochemistry

This procedure was performed by the avidin-biotin-peroxidase complex (ABC) method using ImmunoPure ABC staining kit. The sections were warmed to 42°C for 45 min and deparaffined in xylene, rehydrated with decreasing concentration of ethanol. The endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide solution for 30 min prior to incubation with specific polyclonal rabbit antibodies (PGHS-1, PGHS-2 and Fos at a dilution of 1:200, 1:2,000 and 1:2,000 respectively) for 30 min. Then sections were incubated in a biotinylated goat anti-rabbit IgG solution for 30 min, washed in PBS, and incubated in ABC peroxide reagent for 30 min. Finally, sections were incubated with stable diaminobenzidine dihydrochloride (DAB) solution for 2-7 min, dehydrated in a series of graded ethanol, and cover slipped using Permont.

The following control experiments were performed: 1) omission of the primary antibodies, and 2) substitution with nonspecific immunoglobulin G.

Image Analysis

A digital image analysis system (MCID4, Imaging Research, St. Catherine's, ON, Canada) was used to obtain optical density measurements over the sections. Densitometric measurements were done from 3-5 sections per tissue block per animal.

Statistical Analysis

Data were analyzed by the Student's *t* test, and significance was established at $p < 0.05$. Data were expressed as mean ± SEM. Statistical procedures were accomplished using SigmaStat software (Jandel Scientific, San Rafael, CA).

## Results

### Relationships between PCR Cycles or RT Volume and PCR Yields of PGHS or β-actin cDNA.

In the preliminary experiments we characterized that PCR products for PGHS-1 mRNA was a linear function of PCR cycle number between 31 to 35 cycles of PCR amplification (Fig. 6-1). The PCR products formed was also a linear function of input RT volume from 1 to 5 μl for 33 cycles of PCR amplification (Fig. 6-2). Similarly, a linear relationship between the PCR yield and PCR cycles (28-32 cycles) (Fig. 6-3) or RT volume (1-5 μl) (Fig. 6-4) was characterized for β-actin mRNA. A linear relationship

between the PCR yield and PCR cycles (28-32 cycles) or RT volume (1-5 μl) (data not shown) was characterized for PGHS-2 mRNA. Based on these results, we performed 33 cycles of PCR amplification with 3 μl RT volume for PGHS-1 mRNA, 30 cycles of PCR amplification with 3 μl RT volume for PGHS-2 mRNA, and 30 cycles of PCR amplification with 3 μl RT volume for β-actin mRNA in the followilng studies. The yield of β-actin cDNA was consistant in different tissues. The relative PGHS mRNA levels were compared by the ratio of PGHS mRNA RT-PCR yield/β-actin mRNA RT-PCR yield.

RT-PCR of PGHS-1 and PGHS-2 mRNA

RT-PCR study did not reveal detectable expression of PGHS-2 mRNA in the cerebral cortex and cerebellum of control fetuses, and barely detectable expression in the hypothalamus, brain stem and hippocampus (Fig. 6-5 top panel). However, PGHS-2 mRNA was induced strongly at 30 min after the onset of cerebral ischemia in the hypothalamus, brain stem and hippocampus ($p<0.05$) (Fig. 6-5 bottom panel). In contrast to PGHS-2 mRNA, PGHS-1 mRNA was expressed in all regions of the brain, with highest expression in the brain stem (Fig. 6-6 top panel). PGHS-1 mRNA was not induced by cerebral ischemia (Fig. 6-6 bottom panel).

Immunocytochemistry

In this study, we only focused on staining in some structures in the fetal brain. PGHS-2 like immunoreactive staining was seen in dendrites and cell bodies of neurons. PGHS-2 staining was mainly localized perinuclearly. PGHS-2-ir neurons were observed

in the cerebral cortex, cerebellum, CA1, CA3 and dentate gyrus of the hippocampus, paraventricular nucleus (PVN) of the hypothalamus and nucleus of the solitary tract (NTS) in the brain stem. PGHS-2-ir staining was also observed in endothelial cells and the anterior pituitary gland (Table 6-1). PGHS-2-ir cells were detected at relatively low levels in the NTS of the brain stem, cortex, cerebellum and hippocampus, and relatively high levels in the pituitary, PVN of the hypothalamus and microvasculature. PGHS-2 immunoreactivity was induced in the PVN of the hypothalamus, cerebral cortex, anterior pituitary, hippocampus, NTS of the brain stem, microvasculature at 30 min (figures not shown) and 2 hr (Fig. 6-7 a-f) after a 10-min period of cerebral ischemia. The staining of PGHS-2 at the basal state and 30 min after the ischemic induction was supported by the immunobolt study (Tong and Wood, 1998).

There was extensive distribution of PGHS-1 immunoreactive staining of neuronal cell bodies and dendrites in the fetal brain. PGHS-1-ir cells were observed in the cerebral cortex, cerebellum, hippocampus, PVN of the hypothalamus, NTS of the brain stem, anterior pituitary gland and cerebrovasculature (Table 6-1). The expression of PGHS-1 in the fetal brain was not induced at 30 min after the cerebral ischemia (figures not shown), but it was slightly induced at 2 hr after ischemia in the NTS, PVN (Fig. 6-8 a, b), cerebellum and endothelial cells (figure not shown), and it was not induced in the cortex, hippocampus and the anterior pituitary gland (Fig. 6-8 c-e). The expression of Fos immunoreactivity was relatively low in the endothelial cells, cortex, cerebellum and hippocampus and relatively high in the pituitary, NTS, PVN (Table 6-1). It was induced in all these regions at 2 hr after cerebral ischemia.

Table 6-1. Immunostaining of PGHS-1, PGHS-2 and Fos in different fetal brain regions

| | Time | cortex | cerebellum | NTS | PVN | CA1 | CA3 | dentatus gyrus | pituitary | microvessel |
|---|---|---|---|---|---|---|---|---|---|---|
| | intact | ** | ** | ** | ** | ** | ** | ** | *** | ** |
| PGHS-1 | 30 min | ** | ** | *** | *** | ** | ** | ** | *** | *** |
| | 2 hr | ** | *** | *** | *** | ** | ** | *** | *** | *** |
| | intact | * | * | * | ** | * | * | * | ** | ** |
| PGHS-2 | 30 min | ** | *** | ** | *** | ** | ** | ** | *** | *** |
| | 2 hr | ** | *** | ** | *** | ** | ** | ** | *** | ** |
| | intact | N/A | * | *** | *** | ** | * | ** | *** | ** |
| Fos | 30 min | N/A | ** | *** | *** | *** | *** | *** | ***** | *** |
| | 2 hr | N/A | *** | **** | **** | *** | *** | *** | ***** | *** |

* representive the relative optical density of the staining. NTS: nucleus of the solitary tract of the brain stem. PVN: paraventricular nucleus of the hypothalamus.

## Discussion

The major finding of the present study is that PGHS-2 mRNA, the gene of the inducible form of the prostaglandin endoperoxide synthase which converts arachidonic acid to prostaglandins, was induced at 30 min after global cerebral ischemia. In addition, cerebral ischemia induced PGHS-2 and Fos expressions in neurons, as well as in endothelial cells, indicating that increased prostaglandin productions after the cerebral ischemia can take place not only in neurons but also in non-neuronal cells.

Several non-neuronal structures in the brain showed PGHS immunoreactivity, including the endothelial cells of the microvessels, the ependymal cells lining the lateral ventricles, dorsal aspect of the third ventricle at the level of the thalamus, the floor of the fourth ventricle, and endothelial and stromal cells of the choroid plexus. The basal expression of PGHS-2 revealed by immunoblotting could be derived from these cells in addition to neurons. This study showed that PGHS-2 staining increased in small cerebral vessels. Overall, PGHS-2 induction in non-neuronal cells may be taken as an indication of a ruptured blood-brain barrier during cerebral ischemia (Nedergaard and Diemer, 1987; Koistinaho and Hokfelt, 1997).

The results of this study was consistent with the results in another laboratory showing that the staining of PGHS-2-ir was localized in some structures in the CNS, which may be involved in processing and integration of visceral and special sensory input and in elaboration of the autonomic, endocrine, and behavioral responses (Breder et al. 1995). The relative high level of PGHS-2 immunoreactivity in the PVN, the anterior pituitary gland and microvessels might be due to the stimulation of some fetuses before we

sacrified them. The PGHS-2 immunoreactivity was significantly induced by the cerebral hypoperfusion in this study in all focused regions compared to the basal level. Induction of PGHS-2 has evidently profound effects on the brain and it is likely to affect the outcome of ischemic damage (Miettinen et al. 1997). The increased PGHS-2 activity may control ischemia-induced increase of arachidonic acid levels that may have direct effects on neurotransmitter release and receptors (Katsuki and Okuda, 1995). PGHS, while converting arachidonic acid to eicosanoids, produces free radicals that may contribute to the ischemic neuronal damage (Katsuki and Okuda, 1995).

Our study and other laboratory demonstrated that PGHS-1 immunoreactive neurons were widely distributed in the nuclei or specific regions of the late gestational ovine fetal brain as clusters or in isolation (Norton et al. 1996). PGHS-1-ir neurons were most abundant in forebrain sites involved in complex, integrative functions and autonomic regulation such as the cerebral cortex, PVN of the hypothalamus, and hippocampus. They were also in the structure involved in autonomic regulation, such as NTS. *In situ* hybridization showed that PGHS-1 mRNA is in the corresponding immunoreactive neurons and regions respectively, and it correlated well with the relative number and distribution pattern of PGHS-1 immunoreactive neurons (Norton et al. 1996). Their study also showed that the mRNA encoding PGHS-1 is colocalized in the same cells, indicating that the enzyme is synthesized *de novo* within these cells (Norton et al. 1996).

Breder et al. (1992) suggested that neurons may use prostaglandins as intracellular or perhaps paracrine, but probably not synaptic, mediators in the normal brain. In addition, prostaglandins may facilitate sensory neuropeptide release and sensitize pain

receptors (Ferreira, 1972; Larkin and Williams, 1993). $PGE_2$ binding sites in the fetal brain stem have been localized to some of the nuclei where PGHS immunoreactivity was observed, including the parabrachial nucleus of the pons, and NTS (Tai et al. 1994). PGHS-1 immunoreactivity was also observed in the caudal portion of the NTS which receives general visceral afferents in the human brain (Barr and Kienan, 1988) and suggests that prostanoids could be involved in some autonomic functions. PGHS immunoreactive neurons localized to various regions of the hypothalamus may produce prostanoids that influence neuropeptide release and various regulatory functions of the autonomic nervous system.

The fos/jun immediate early genes (IEGs) are induced in the brain by a variety of physiological and pathological stimuli. Cerebral ischemia is known to induce the expression of several IEGs, including c-fos and c-jun, which subsequently regulate a number of late effector genes (Kindy et al. 1991; Onodera et al. 1989). The induction of IEGs is rapid and transient. Kindy and colleagues (1991) demonstrated that binding of the Fos:Jun complex to the AP-1 domain peaked 2 h after ischemic injury in the gerbil. Transient global ischemia after bilateral common carotid artery occlusion in gerbils (Ikeda et al. 1990) induced the expression of c-fos mRNA in dentate gyrus neurons from 15-60 min after insult and in CA1 and CA3 neurons after 30 to 60 min. Ceccatelli et al. (1989) demonstrated that different stressful stimuli can induce c-fos in different subpopulations of cells in the PVN and SON of the hypothalamus.

The expression of c-fos during brain development is different under basal conditions. High levels of c-fos were seen throughout the CNS during embryonic stage

and the expression was limited to some areas during later stages (Caubert, 1989). Basal levels of c-fos are very low in adult animals. The expression after stimuli is different in the adult and fetal animals (Ruppert and Wille, 1987). The immature brain is highly responsive to hypoxia-ischemia at the level of gene expression, involving at least three different rapid response system (Gubits et al. 1993). In our study, the Fos staining was observed on all focused fetal tissues of intact animals and it was induced at 2 hr after cerebral hypoperfusion. We did not observe the Fos immunoreactivity induction at the PVN and NTS compared to that in the intact animals. The explanation of the relative higher staining of Fos in the intact group is that some fetuses might be stimulated before they were sacrificed. The inducibility of Fos has been used as a tool to study neuronal activation in different systems in the brain and it represents a sensitive marker of cellular responses to ischemia (Gubits et al. 1993). The mechanisms underlying the induction of Fos after brain ischemia are yet to be elucidated. Its detection has led to a further understanding of different systems in the CNS and allows the mapping of neuronal populations activated by a variety of external stimuli.

In summary, immunocytochemistry and RT-PCR studies indicated that PGHS was synthesized *de novo* within the fetal brain during cerebral hypoperfusion and its products, prostanoids, may act as autocrine/paracrin factors to regulate diverse physiological functions, such as cardiovascular and endocrine systems. PGHS-2 and Fos inducibility by cerebral ischemia together with the absence of PGHS-1 induction indicated that, PGHS-2 is the major prostaglandin endoperoxide synthase contributing to prostanoid synthesis in the late-gestation fetal sheep brain.

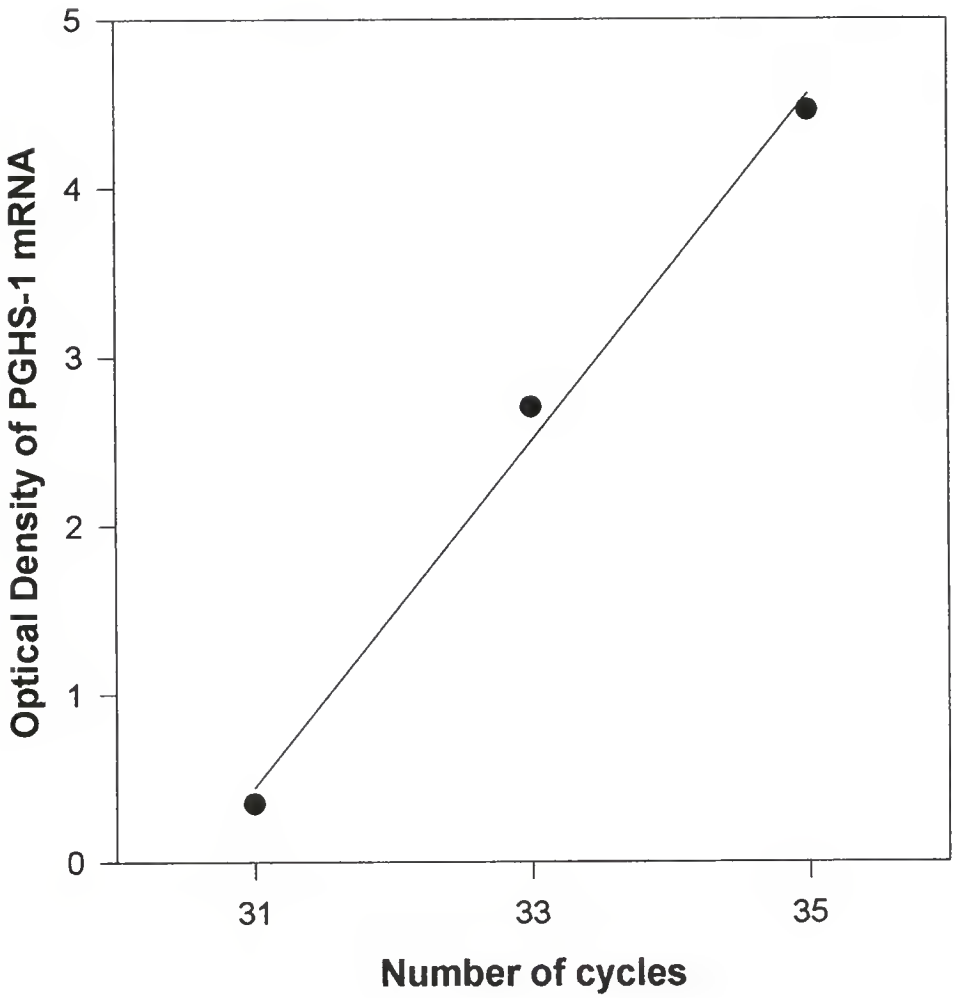



Figure 6-1. Relationship between PCR cycles and PCR yields of PGHS-1 cDNA. PCR products formed is a linear function of PCR cycle number between 31 to 35 cycles of PCR amplification.

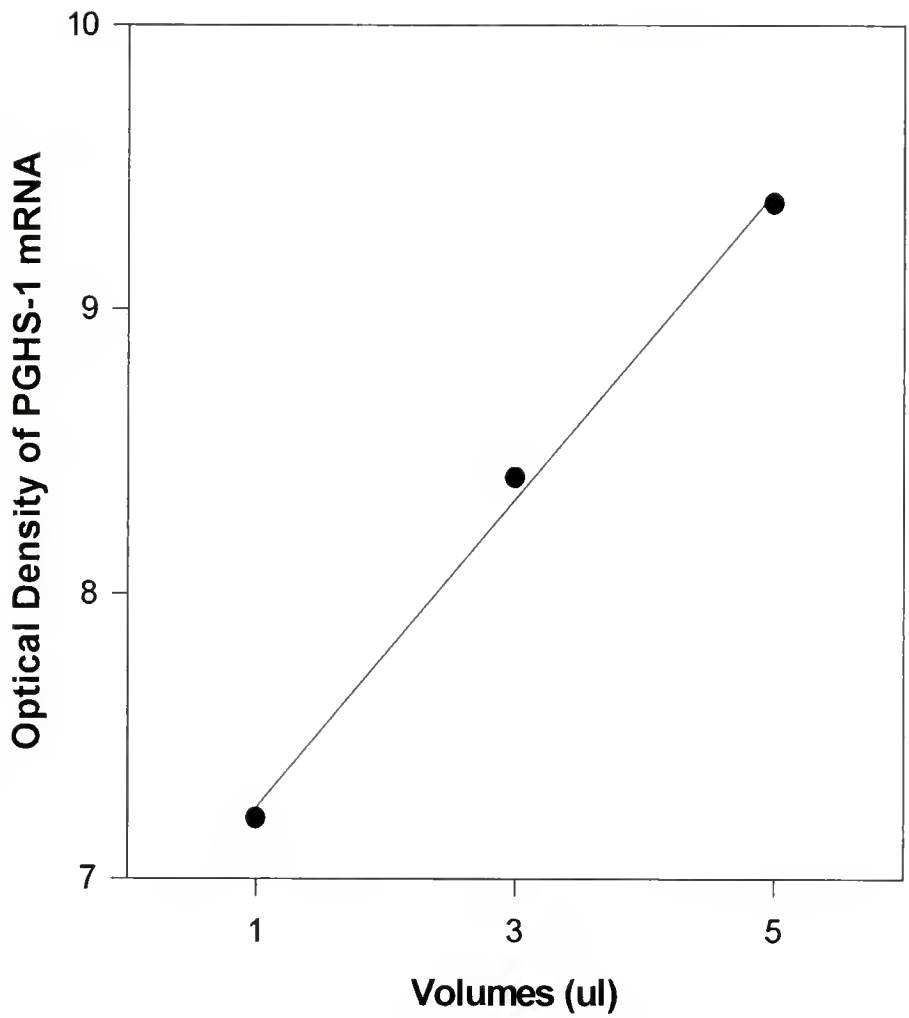



Figure 6-2. Relationship between RT volume in PCR reaction and PCR yields of PGHS-1 cDNA. PCR products formed is a linear function of RT volume between 1 to 5 µl of PCR amplification.

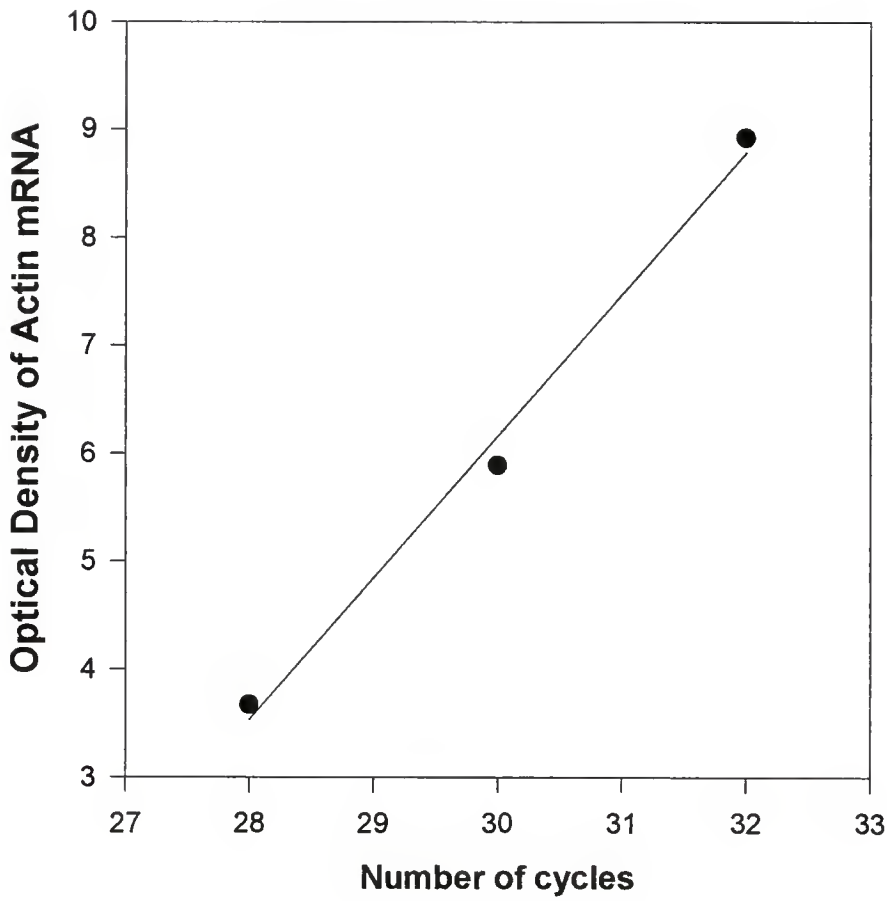



Figure 6-3. Relationship between PCR cycles and PCR yields of β-actin cDNA. PCR products formed is a linear function of PCR cycle number between 28 to 32 cycles of PCR amplification.

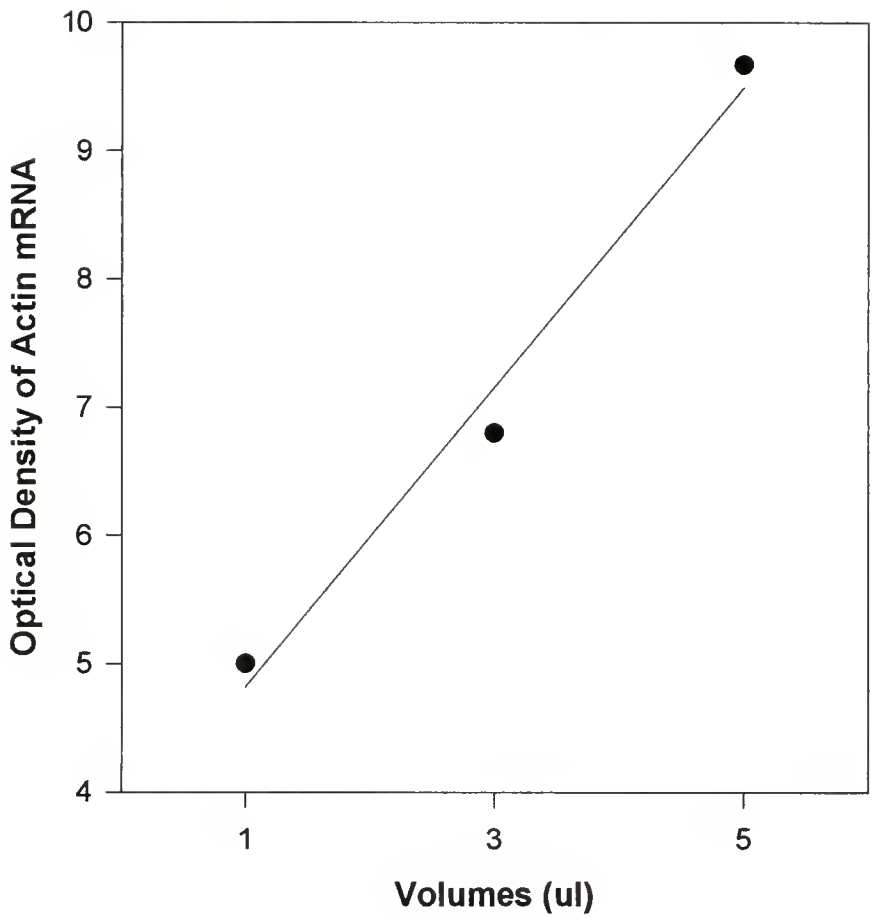
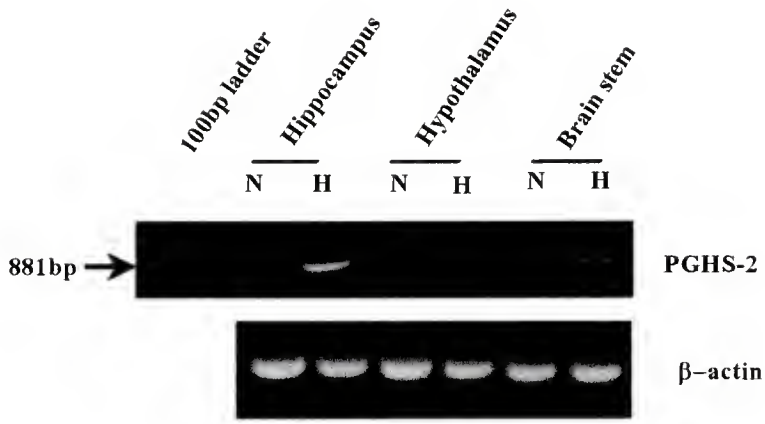



Figure 6-4. Relationship between RT volume in PCR reaction and PCR yields of β-actin cDNA. PCR products formed is a linear function of RT volume between 1 to 5 µl of PCR amplification.


100bp ladder
Hippocampus
Hypothalamus
Brain stem
N H N H N H
881bp
PGHS-2
β-actin

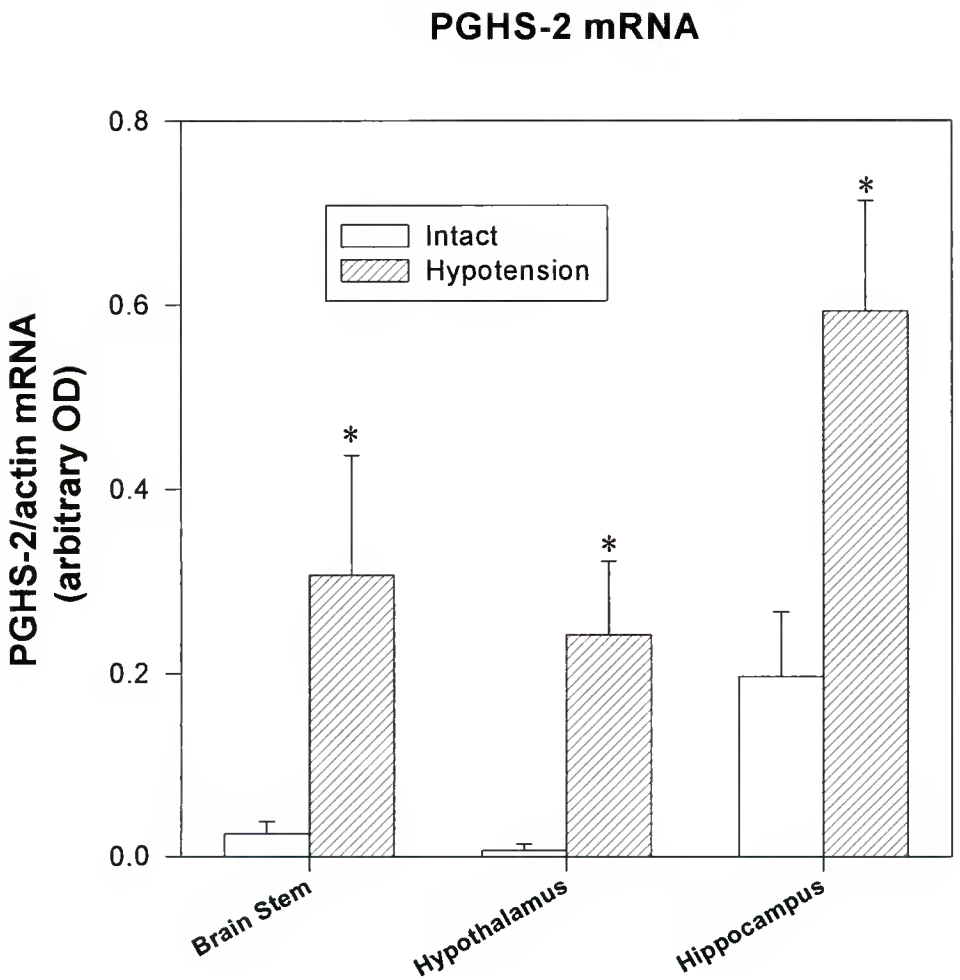
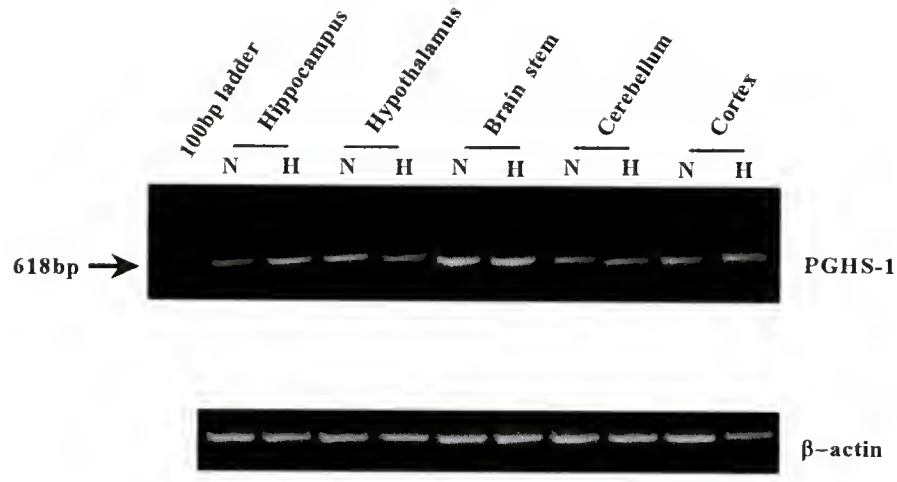

PGHS-2 mRNA
PGHS-2/actin mRNA (arbitrary OD)
0.8
0.6
0.4
0.2
0.0
Intact
Hypotension
*
*
*
Brain Stem
Hypothalamus
Hippocampus

Figure 6-5. The change of PGHS-2 mRNA in brain tissues of denervated fetuses following cerebral hypoperfusion. Top: representative ethidium bromide stained gels showing the RT-PCR DNA products which correspond to PGHS-2 (881 bp) and β-actin (0.5 kb) mRNAs. The 100 bp DNA ladder is on the left side. N: represents the tissue from intact aminal. H: represents the tissue from 30 min after a 10-min period of hypoperfusion animal. Bottom: quantification of the DNA bands corresponding to the PGHS-2 mRNA. Data are expressed as the ratio of PGHS-2 mRNA/β-actin mRNA in each brain tissue. Values are means ± SE, n=5, *$p<0.05$ difference between groups.


100bp ladder
Hippocampus
Hypothalamus
Brain stem
Cerebellum
Cortex
N H N H N H N H N H
618bp
PGHS-1
β-actin

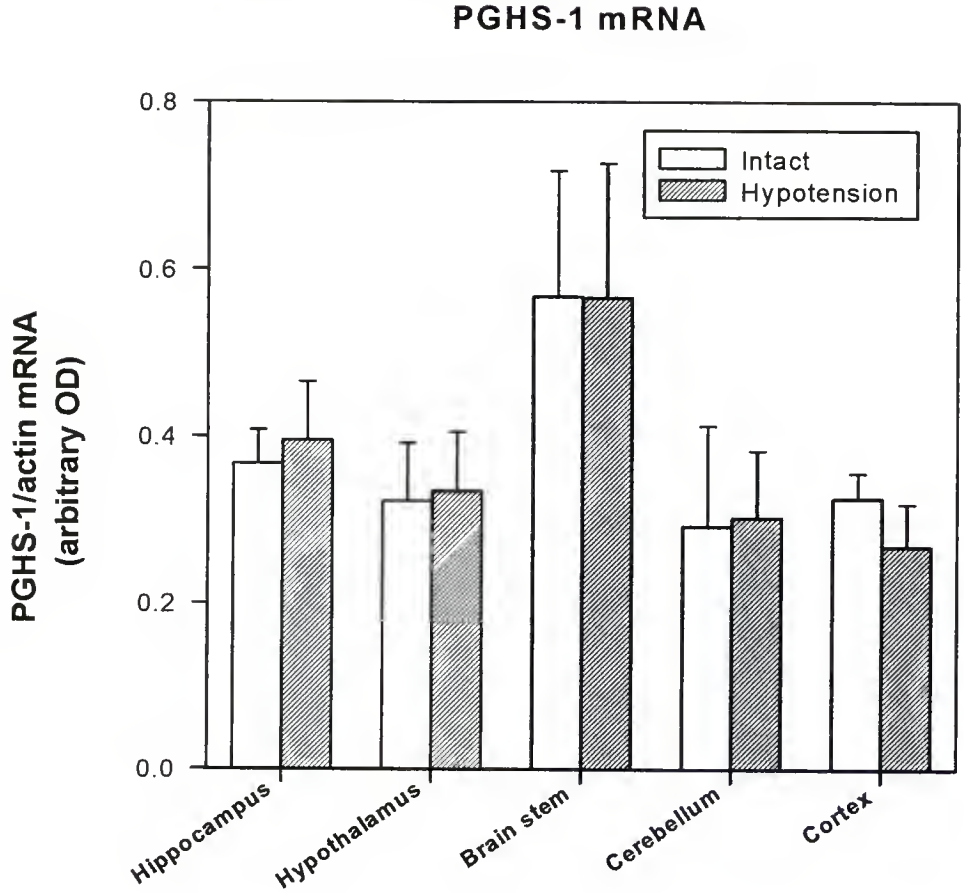

PGHS-1 mRNA
PGHS-1/actin mRNA
(arbitrary OD)
0.8
0.6
0.4
0.2
0.0
Intact
Hypotension
Hippocampus
Hypothalamus
Brain stem
Cerebellum
Cortex

Figure 6-6. The change of PGHS-1 mRNA in brain tissues of denervated fetuses following cerebral hypoperfusion. Top: representative ethidium bromide stained gels showing the RT-PCR DNA products which correspond to PGHS-1 (618 bp) and β-actin (0.5 kb) mRNAs. The 100 bp DNA ladder is on the left side. N: represents the tissue from intact aminal. H: represents the tissue from 30 min after a 10-min period of hypoperfusion animal. Bottom: quantification of the DNA bands corresponding to the PGHS-1 mRNA. Data are expressed as the ratio of PGHS-1 mRNA/β-actin mRNA in each brain tissue. Values are means ± SE, n=5.

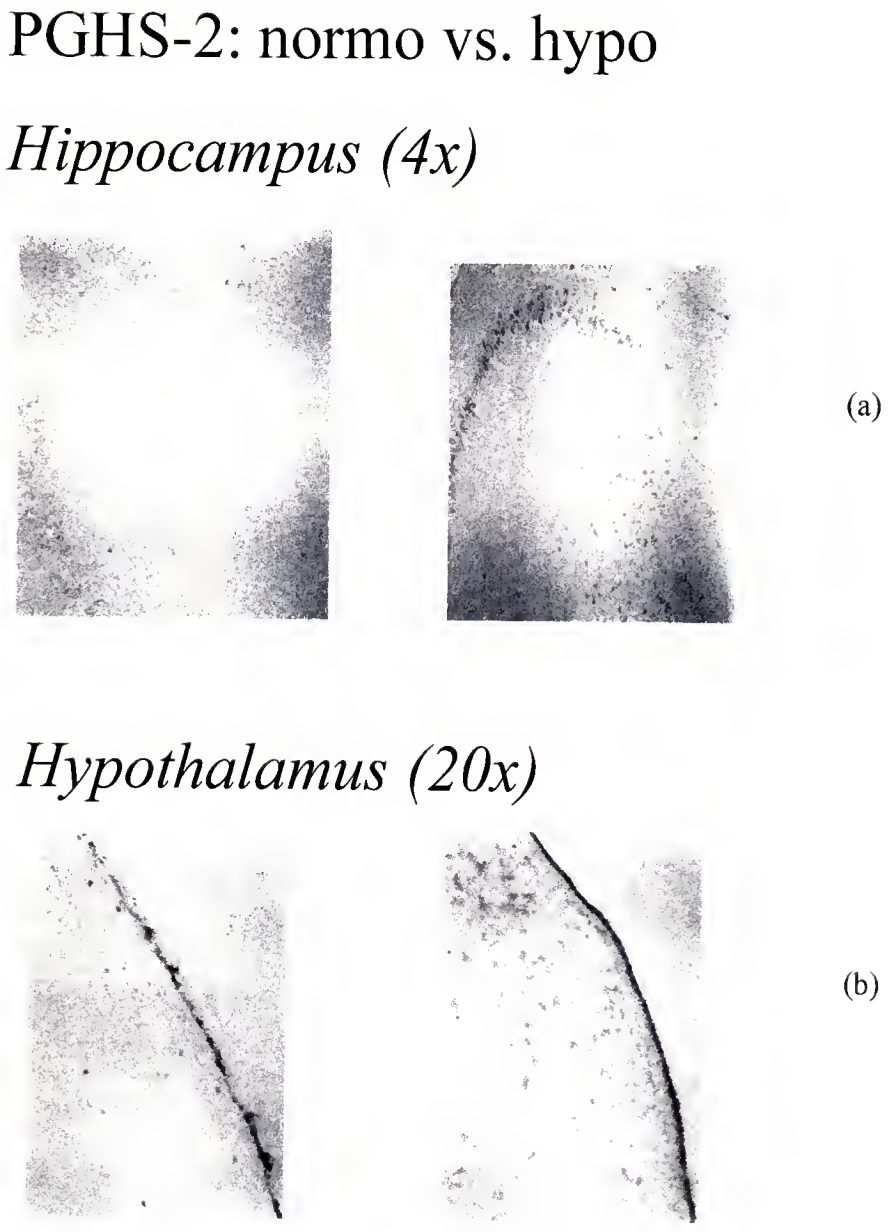
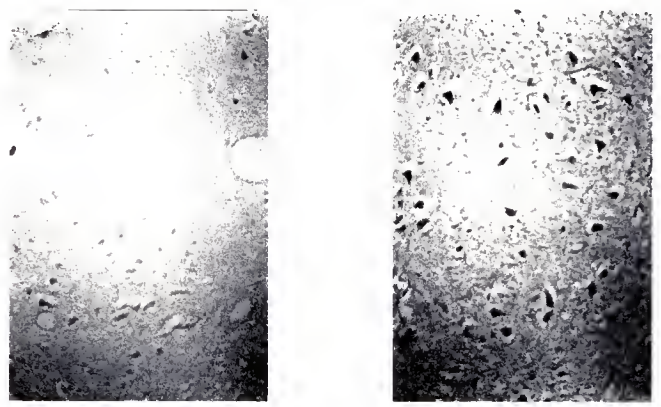



Figure 6-7. PGHS-2 immunoreactivity in the fetal brain from the intact animal (left side) and 2 h after cerebral hypoperfusion animal (right side). Induction of PGHS-2 immunoreactivity is seen in the hippocampus (a), PVN (b), ventrolateral medulla (c), cerebral cortex (d), anterior pituitary gland (e), and cerebral vasculature (f).

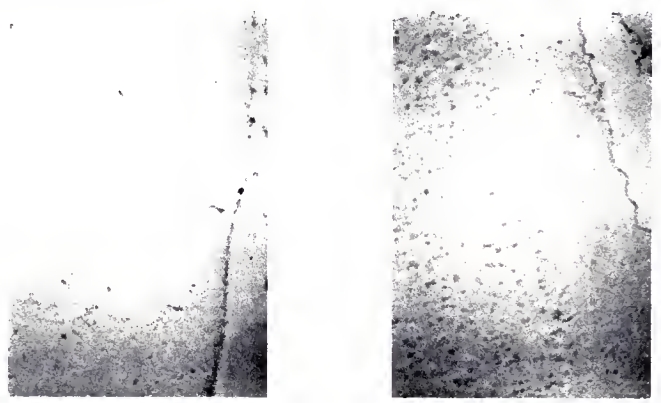
PGHS-2 normo vs. hypo

*Ventrolateral Medulla (10x)*



(c)

*Cerebral Cortex (10x)*



(d)

Figure 6-7 (continued) PGHS-2 immunoreactivity in the fetal brain from the intact animal (left side) and 2 h after cerebral hypoperfusion animal (right side). Induction of PGHS-2 immunoreactivity is seen in the hippocampus (a), PVN (b), ventrolateral medulla (c), cerebral cortex (d), anterior pituitary gland (e), and cerebral vasculature (f).

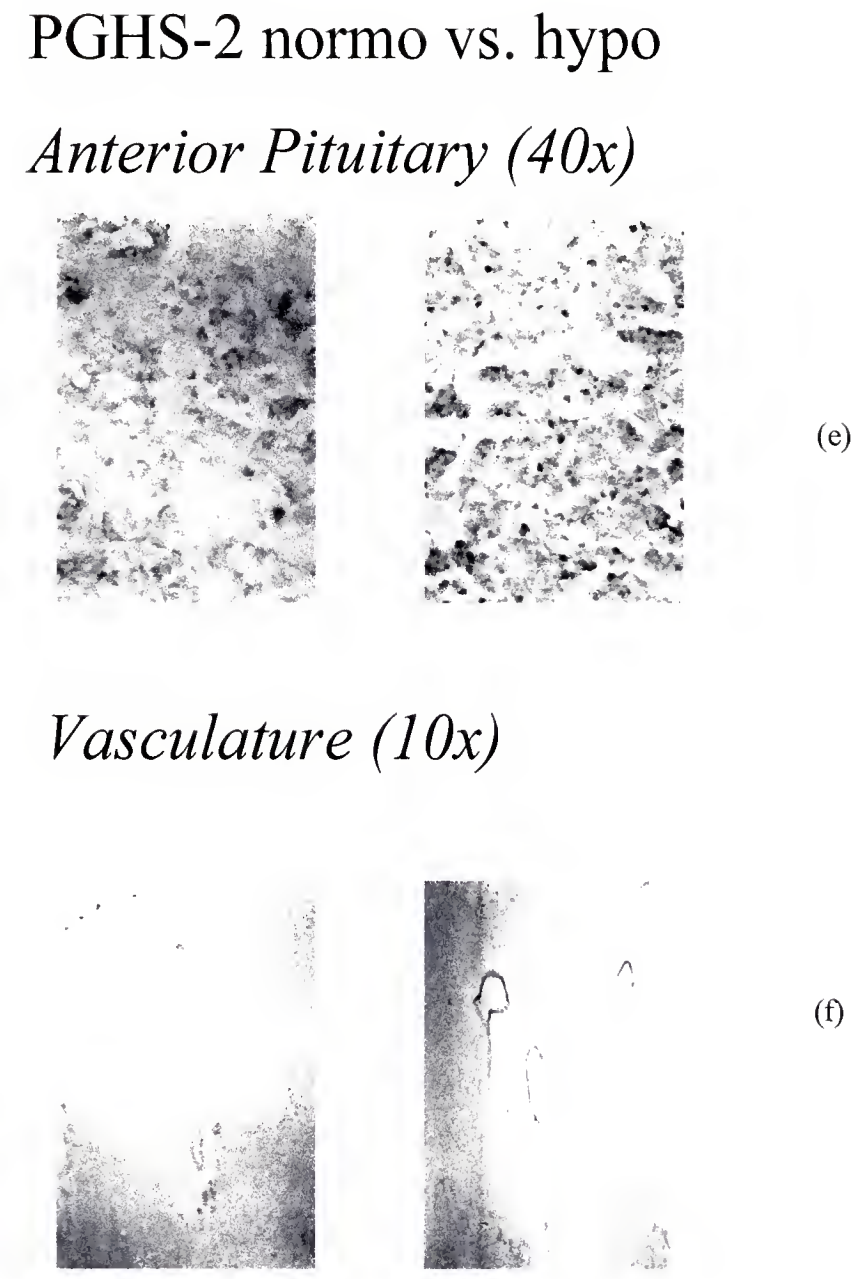



Figure 6-7 (continued) PGHS-2 immunoreactivity in the fetal brain from the intact animal (left side) and 2 h after cerebral hypoperfusion animal (right side). Induction of PGHS-2 immunoreactivity is seen in the hippocampus (a), PVN (b), ventrolateral medulla (c), cerebral cortex (d), anterior pituitary gland (e), and cerebral vasculature (f).

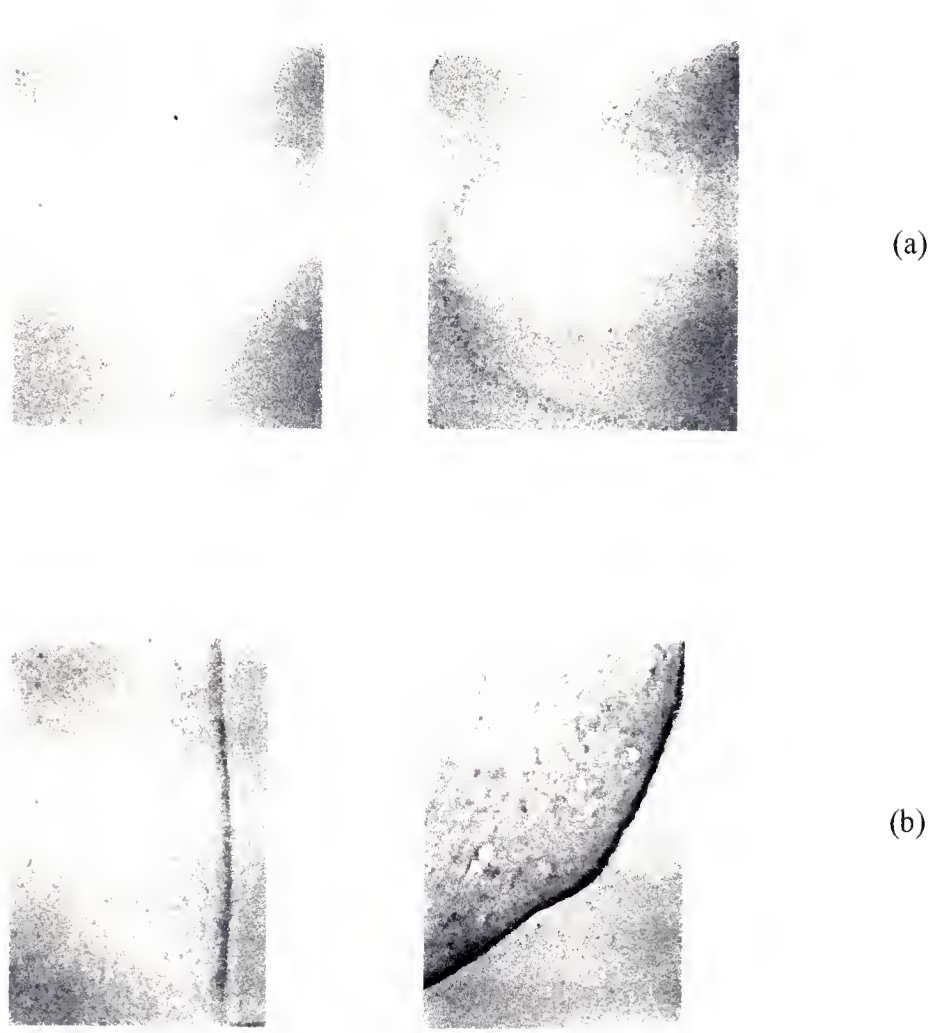



Figure 6-8. PGHS-1 immunoreactivity in the fetal brain from the intact animal (left side) and 2 h after cerebral hypoperfusion animal (right side). The immunoreactive PGHS-1 is seen in the hippocampus (a), PVN (b), ventrolateral medulla (c), cerebral cortex (d), and anterior pituitary gland (e)

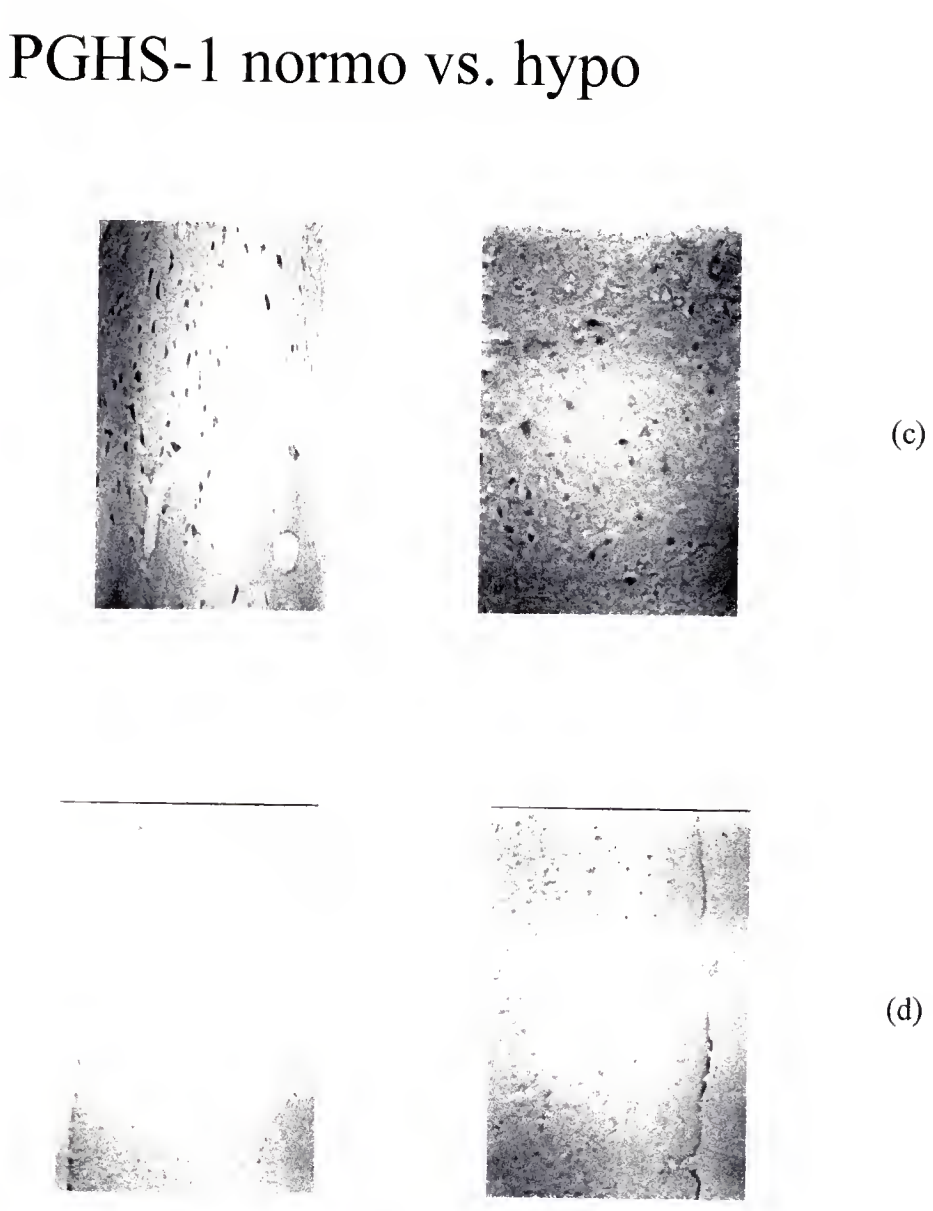



Figure 6-8 (continued) PGHS-1 immunoreactivity in the fetal brain from the intact animal (left side) and 2 h after cerebral hypoperfusion animal (right side). The immunoreactive PGHS-1 is seen in the hippocampus (a), PVN (b), ventrolateral medulla (c), cerebral cortex (d), and anterior pituitary gland (e)

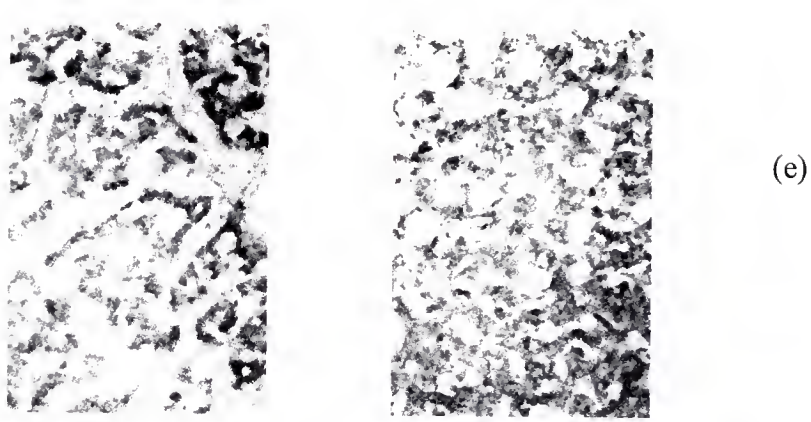



Figure 6-8 (continued) PGHS-1 immunoreactivity in the fetal brain from the intact animal (left side) and 2 h after cerebral hypoperfusion animal (right side). The immunoreactive PGHS-1 is seen in the hippocampus (a), PVN (b), ventrolateral medulla (c), cerebral cortex (d), and anterior pituitary gland (e).

# CHAPTER 7
# SUMMARY AND CONCLUSIONS

The clinical use of fetal surgery for correction of fetal congenital anomalies has developed rapidly. At the same time, the clinical use of prostaglandins and prostaglandin synthase inhibitors has also increased. While surgical and medical treatment of fetuses has become more common, our basic understanding of the mechanism controlling fetal blood pressure and fetal responses to stress is incomplete. Improvement of our basic understanding of fetal homeostasis is the key to improve clinical management and treatment strategies. The enzyme prostaglandin endoperoxide synthase has been localized in human, rat, mouse and sheep brain during conditions of inflammation and injury. The induction of the gene of the inducible prostaglandin endoperoxide synthase leads to high levels of prostanoids release which contribute to a variety of physiological and pathological processes via autocrine and paracrine fashions, including inflammation, smooth muscle tone, hemostasis, thrombosis, parturition, cardiovascular and endocrine functions.

Fetal sheep respond to hypotension with reflex hormonal and hemodynamic responses which return blood pressure to normal levels. The reflex vasomotor and heart rate responses and the reflex hormonal responses are mediated by both baroreceptor and chemoreceptor reflexes. The arterial baroreceptor regulates the minute to minute change

in arterial blood pressure. Hypotension increases the secretion of ACTH, AVP and cortisol which is mediated by arterial baroreceptor and chemoreceptor. However, these reflex responses are only 50% inhibited by peripheral baroreceptor and chemoreceptor denervation (Wood, 1989). Prostanoids generated in the central nervous system appear to contribute a portion of the hormonal reflex responses since intracerebroventricular infusion of prostaglandin $E_2$ or U46619 (a thromboxane $A_2$ mimetic) into the carotid artery stimulates ACTH secretion (Brooks, 1992; Wood et al. 1993). Thus, this research project had the specific goal of determining: (1) if prostanoids could be produced in the fetal brain in response to cerebral hypoperfusion; (2) if prostaglandin endoperoxide synthase expression could be induced by cerebral hypoperfusion; (3) whether these prostanoids could mediate the ACTH and AVP responses to hypotension.

First, the arterial hypotension produced by vena caval occlusion significantly stimulated fetal ACTH, AVP, and cortisol secretion, and the ACTH and AVP responses were attenuated by carotid sinus denervation. This result confirmed that the hormonal reflex responses to arterial hypotension are mediated by arterial baroreceptors and chemoreceptors, but are not completely eliminated by the denervation procedure. In this study, we pretreated fetuses with the cyclooxygenase inhibitor indomethacin to test the hypothesis that endogenous prostanoids might mediate a portion of this reflex responses. We found that arterial plasma concentrations of prostaglandin $E_2$ and thromboxane $B_2$ (the stable metabolite of thromboxane $A_2$) were significantly decreased by indomethacin treatment. One interesting observation of this study is that the ACTH and AVP responses to hypotension were partially inhibited by indomethacin in intact fetuses, but these

responses were not further attenuated by indomethacin in denervated fetuses. This suggests that the endogenous production of prostanoids modulates the ACTH and AVP reflex responses to arterial hypotension and the action of the prostanoids must be on the afferent pathways or on central elements activated by the afferent pathways.

The hypotension produced by vena caval occlusion decreases blood pressure throughout the body. Next, we wanted to test the hypothesis that cerebral hypoperfusion could produce the same reflex hormonal responses. Brachiocephalic occlusion decreases perfusion pressure to the fetal brain and hence reduces cerebral blood flow significantly. We found that the ACTH and AVP responses to cerebral hypoperfusion were similar to that of arterial hypotension. The magnitude of these responses was attenuated by carotid sinus denervation but not completely eliminated. Indomethacin treatment attenuated the ACTH and AVP responses to cerebral hypoperfusion in intact fetuses but not in denervated fetuses. This suggests that the reduction in cerebral blood flow may stimulate endogenous prostanoids production in the fetal brain which in turn modulates the hormonal and cardiovascular responses due to baro- and chemo-receptor stimulation. Another observation from this study is that indomethacin decreases the reduction in cerebral blood flow, suggesting that indomethacin has beneficial effect on fetal cerebral hypoperfusion by blocking vasoconstrictor prostanoids in the fetal brain.

Since prostanoids can be produced in most tissues and act on many organs, the results from the above two studies do not distinguish the source of prostanoids from the central nervous system or from peripheral tissues. The microdialysis study showed that prostanoids increased in sagittal sinus blood and in brain interstitial fluid during cerebral

hypoperfusion, suggesting that prostanoids may be produced in the cerebrovasculature and in neurons. To further identify the cell types responsible for the production of prostanoids in the fetal brain, we used immunocytochemistry study. Prostaglandin endoperoxide synthase (PGHS), the key enzyme of prostanoids biosynthesis, was localized in the endothelial cell of small blood vessels, neurons of the cerebral cortex, cerebellum, CA1, CA3, and dentatus gyrus of the hippocampus, paraventricular nucleus (PVN) of the hypothalamus, the nucleus of the solitary tract (NTS) of the brain stem, and other non-neuronal cells, including the anterior pituitary gland, dorsal aspect of the third ventricle at the level of the thalamus, and the floor of the fourth ventricle. The staining of inducible PGHS-2 in the CNS suggests that it may be involved in processing and integration of visceral and special sensory input and in elaboration of the autonomic, endocrine, and behavioral responses (Breder et al. 1995). The constitutive PGHS-1-ir staining in the brain suggests that it may be involved in integrative functions, some autonomic functions, and facilitating sensory neuropeptide release (Breder et al. 1992; Larkin and Williams, 1993). We also observed that staining of PGHS-2-ir, but not PGHS-1, is induced by cerebral hypoperfusion.

Finally, based on the finding that prostanoids are produced in the fetal brain during cerebral hypoperfusion and immunoreactive PGHS-2 is induced, we wanted to determine the mechanism by which prostanoids in the brain modulate the ACTH and AVP secretion in response to hypotension. Western blot analyses demonstrated that PGHS-2 protein in fetal brain tissues is induced by cerebral hypoperfusion and it is associated with the "suicide" inactivation of prostaglandin endoperoxide synthase. On the other hand, PGHS-

1 protein does not change in response to cerebral hypoperfusion. RT-PCR analyses further demonstrated that in fetal brain tissues, PGHS-2 mRNA, but not PGHS-1, is induced by cerebral hypoperfusion. Fos immunocytochemistry study showed increases in response to cerebral ischemia. It is known that the PGHS-2 gene contains an activator protein-1 (AP-1) binding site on the promoter sequence (Xie et al. 1993), suggesting that PGHS-2 gene may be transcriptionally regulated by c-fos.

In summary (Fig. 7-1), these studies indicate that hypotension decreases cerebral blood flow, which in turn induces the mRNA and protein expression of prostaglandin endoperoxide synthase-2 within the fetal brain. The enzyme PGHS-2 is the major prostaglandin endoperoxide synthase contributing to the prostanoids synthesis. The induction of PGHS-2 increases prostanoids biosynthesis. PGHS is located in the PVN and NTS where the baroreceptor and chemoreceptor signals enter and relay in the central nervous system. These elevated levels of prostanoids may act as a neuromodulator in the hypothalamus and/or the anterior pituitary gland in an autocrine/paracrine fashion to stimulate plasma corticotropin releasing factor (CRF), AVP, and ACTH secretions. CRF and AVP from the fetal hypothalamus stimulate the ACTH secretion from the anterior pituitary gland. And ACTH stimulates the cortisol secretion from the adrenal cortex. Increased levels of these hormones compensate for the hypotension and return blood pressure and blood volume to normal levels. AVP is involved in both immediate and long-term blood pressure control, since it is a potent vasoconstrictor and a promotor of antidiuresis acting at the kidney. Increased cortisol secretion can maintain vascular reactivity of the vasculature to direct vasoconstrictors such as AVP and increase the

conservation of sodium by the kidney. Therefore, this study has reveled another pathway to regulate the hormonal and cardiovascular reflex responses to hypotension in late-gestation fetal sheep in addition to baroreceptors and chemoreceptors reflex responses.

Further studies are required to investigate the prostanoids receptors involved in the modulation of hormonal reflex responses in the central nervous system in response to cerebral hypoperfusion. The gene expression of PGHS at different time points after cerebral hypoperfusion, and some other potential regulatory component(s) responsible for the reflex responses to hypotension need to be uncovered.

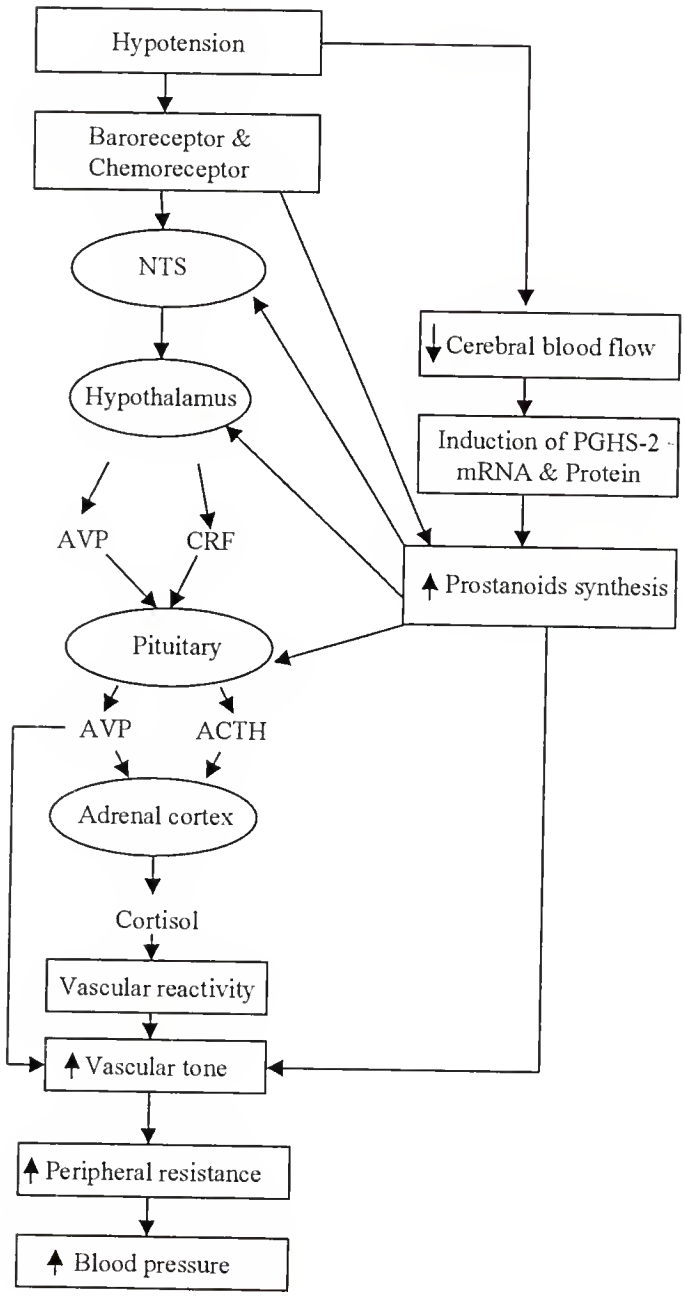



Figure 7-1. Proposed regulatory mechanism of the prostaglandins on the fetal hypothalamus-pituitary-adrenal axis in response to hypotension.

# REFERENCES


Backofen, J.E., Keohler, R.C., Harris, A.P., Rogers, M.C., Traystman, R.J., and Jones, M.D. Efficacy of cushing response during development in sheep. *Am. J. Physiol.* 261:H575-H582, 1991.

Bada, H.S., Green, R.S., Pourcyrous, M., Leffer, C.W., Korones, S.B., Magill, H.L., Arheart, K., Fitch, C.W., Anderson, G.D., Somes, G., Tullis, K., Campbell, J. Indomethacin reduces the risks of severe intraventricular hemorrhage. *J. Pediatrt.* 115:631-637, 1989.

Bannwarth, B., Netter, P., Lapicque, F., Pere, P., Gaucher, A. Plasma and cerebrospinal fluid concentrations of indomethacin in humans. *Eur. J. Clin. Pharmacol.* 38:343-346, 1990.

Barr, M.L. and Kienan, J.A. *The human nervous system. An Anatomical viewpoint.* 5th ed. J.B. Lippincott Co. Philadelphia. PA. 1988.

Bartter, F.C., and Fourman, P. The different effects of alsosteron-like steroids and hydrocortisone-like steoids on urinary excretion of potassium and acid. *Metabolism.* 11:6-20, 1962.

Bartter, F.C., Millls, I.H., and Gann, D.S. Increase in alsosterone secretion by carotid artery constriction in the dog and its prevention by thyrocarotid arterial junction denervation. *J. Clin. Invest.* 39:1330-1336, 1960.

Bernardini, R., Chiarenza, A., Calogero, A.E., Gold, P.W., and Chrousos, G.P. Arachidonic acid metabolites modulate rat hypothalamic corticotropin releasing hormone in vitro. *Neuroendocrinology.* 50:708-716, 1989.

Bie, P. Quantitative aspects of renal actions of vasopressin. *Acta Physiol. Scand.* 139:28-32, 1990.

Borgeat, P. and Samuelsson, B. Metabolism of arachidonic acid in polymophonuclear leukocytes: structural analysis of novel hydroxylated compoents. *J. Biol. Chem.* 254:2643-2646, 1979.

Bradford, M.A. Rapid and sensitive method for the quatitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem.* 72:248-254, 1976.

Breder, C. D., DeWitt, D. L., and Kraig, R. P. Characterization of inducible cyclooxygenase in rat brain. *J. Comp. Neurol.*. 355:296-315, 1995.

Breder, C. D., Smith, W. L., Raz, A., Masferrer, J.. Seibert, K., Needleman, P., and Saper, C. B. Distribution and characterization of cyclooxygenase immunoreactivity in the ovine brain. *J. Comp. Neurol.* 322:409-438, 1992.

Breuhaus, B.A. and Chimoskey, J.E. $PGE_2$ does not act at carotid sinus to raise arterial pressure in conscious sheep. *Am. J. Physiol.* 245:H1007-H1012, 1983.

Breuhaus, B.A. and Chimoskey, J.E. Mechanisms of tachycardia caused by intracarotid $PGE_2$ in conscious ewes. *Proceedings of the Society for Experimental Biology and Medicine Med.* 180:353-358, 1985.

Breuhaus, B.A., Demarest, K.T., and Chimosky, J.E. Comparison of intraventricular and intracarotid infusions of $PGE_2$ in conscious sheep. *Am. J. Physiol.* 256:R685-R693, 1989.

Brezinski, M.E., Yanagisawa, A. and Lefer, A.M. Cardioprotective actions of specific thromboxane receptor antagonist in acute myocardial ischemia. *J. Cardiovasc. Pharmacol.* 9:65-71, 1986.

Brooks, A.N. Prostaglandin $E_2$ stimulates adrenocorticotrophin and cortisol secretion via a hypothalamic site of action in fetal sheep. *J. Develop. Physiol.* 18:173-177, 1992.

Brooks, A.N., and Gibson, F. Prostaglandin $E_2$ enhances AVP-stimulated but not CRF-stimulated ACTH secretion from cultured fetal sheep pituitary cells. *J. Endocrinology.* 132:33-38, 1992.

Brooks, D.P., Share, L.,and Crofton, J.T. Role of brain prostaglandins in the control of vasopressin secretion in the conscious rat. *Endocrinology.* 118:1716-1722, 1986.

Brooks, D.P., Share, L., Crofton, J.T., and Nasjletti, A. Central indomethacin enhaces volume-dependent vasopressin release. *Am. J. Physiol.* 247:R1017-R1021, 1984.

Burch, R.M., Wise, W.C., Halushka, P.V. Prostaglandin-independent inhibition of calcium transport by nonsteroidal anti-inflammatory drugs: Differential effects of carboxylic acids and piroxicam. *J. Pharmacol. Exp. Ther.* 227:84-91, 1983.

Campbell, W.B., and Halushka, P.V. Lipid-derived autacoids: eicosanoids and platelet-activating factor. In: *Goodman and Gilman's The Pharmacological Basis of*

*Therapeutics.*, edited by A.G. Gilman, T.W. Rall, A.S. Nies, and P. Taylor. McGraw-Hill, Health Professcial, New York: Pergamon Press, 1996, pp, 601-617.

Caubert, J. C-fos proto-oncogene expression in the nervous system during mouse development. *Mol. Cell Biol.* 9:2269-2272, 1989.

Cazevieille, C., Muller, A., Meynier, F., Dutrait, N., Bonne, C. Protection by prostaglandins from glutamate toxicity in cortical neurons. *Neurochem. Int.* 24:395-398, 1994.

Ceccatelli, S., Villar, M.J., Goldstein, M. and Hokfelt, T. Expression of c-fos immunoreactivity in transmitter-characterized neurons after stress. *Proc. Natn. Acad. Sci. U. S. A.* 86:9569-9573, 1989.

Chau, L.Y., and Tai, H.H. Release of arachidonate from diglyceride in human platelets requires the sequatial action of a diglyceride lipase and a monoglyceride lipase. *Biochem. Biophys. Res. Commun.* 100:1688-1695, 1981.

Chemtob, S., Beharry, K., Rex, J., Varma, D.R., and Aranda. J.V. Prostanoids determine the range of cerebral blood flow autoregulation of newborn piglets. *Stroke*. 21:777-784, 1990a.

Chemtob, S., Beharry, K., Rex, J., Varma, D.R., and Aranda. J.V. Changes in cerebrovascular prostaglandins and thromboxane as a function of systemic blood pressure. *Circ. Res.* 67:674-682, 1990b.

Chemtob, S., Laudignon, N., Beharry, K., Rex, J., Wolfe, L, Varma, D.R., and Aranda, J.V. Effects of prostaglandins and indomethacin on cerebral blood flow and cerebral oxygen consumption of conscious newborn piglets. *Dev. Pharmacol. Ther.* 14:1-14, 1990c.

Chen, H.G. and Wood, C.E. Reflex control of fetal arterial pressure and hormonal responses to slow hemorrhage. *Am. J. Physiol.* 262:H225-H233, 1992.

Chen, S.T., Hsu, C.Y., Hogan, E.L., Halushka, P.V., Linet, O.I., Yatsu, F.M. Thromboxane, prostacyclin, and leukotrienes in cerebral ischemia. *Neurology.* 36:466-470, 1986.

Chen, Y.N. P., Bienkowshi, M.J., and Marnett, L.J. Controlled tryptic digestion of prostaglandin H synthase. *J. Biol. Chem.* 262:16892-16899, 1987.

Chen, Y.N. P., and Marnett, L.J. Heme prosthetic group required for acetylation of prostaglandin H synthase by aspirin. *FASEB J.* 3:2294-2297, 1989.

Chiu, R., Boyle, W.J., Meek, J., Smeal, T., Hunter, T., and Karin, M. The c-fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. *Cell.* 54:541-552, 1988.

Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry.* 16:156-159, 1987.

Cohen-Luria, R., Rimon, G., and Moran, A. $PGE_2$ inhibits Na, K-ATPase activity and ouabain binding in MDCK cells. *Federation Proc.* 30-41, 1992.

Coleman, R.A., Smith, W.L., and Narumiya, S. International union of pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol. Rev.* 46:205-229, 1994.

Collaco-Moraes, Y., Aspey, B., Harrison, M., and de Belleroche, J. Cyclooxygenase-2 messenger RNA induction in focal cerebral ischemia. *J. Cereb. Blood Flow Metab.* 16:1366-1372, 1996.

Cowley, A.W.J., Liard, J.F., and Guyton, A.C. Role of the baroreceptor reflex in daily control of arterial blood pressure and other variables in dogs. *Circ. Res.* 32:564-576, 1973.

Coyle, M.G., Oh, W., Petersson, K.H., and Stonestreet, B.S. Effects of indomethacin on brain blood flow, cerebral metabolism, and sagittal sinus prostanoids after hypoxia. *Am. J. Physiol.* 269:H1450-H1459, 1995.

Cudd, T.A. Vagal nerve blockade does not prevent the ACTH, cortisol, and blood gas responses to infusions of thromboxane $A_2$ mimetic. *FASEB J.* 11:2819, 1997. (Abstract)

Cudd, T.A., and Wood, C.E. Does intracarotid $PGE_2$ increase plasma ACTH concentrationn in conscious adult ewes? *Am. J. Physiol.* 261:E395-E401, 1991.

Cudd, T.A., and Wood, C.E. Prostaglandin $E_2$ release ovine fetal ACTH from a site not perfused by the carotid vasculature. *Am. J. Physiol.* 263:R136-R140, 1992.

Cudd, T.A., and Wood, C.E. Prostanoid cascade inhibition prevents the cardiovascular and adrenocorticotropic responses to mineral acid infusion in conscious sheep. *Am. J. Physiol.* 264:R1235-R1241, 1993.

Cudd, T.A., and Wood, C.E. Thromboxane $A_2$ receptor antagonism prevents the hormonal and cardiovascular responses to mineral acid infusion. *Am. J. Physiol.* 267:R1235-R1240, 1994.

Davidson, E.M., Doig, M.V., Ford-Hutchinson, A.W. and Smith, M.J. Prostaglandin and thromboxane production by rabbit polymorphonuclear leukocytes and rat macrophages. *Adv. Prostaglandin Thromboxane Res.* 8:1661-166, 1980.

Day, R.O., Graham, G.G., Williams, K.M., Champion, G.D., de Jager, J. Clinical pharmacology of non-steroid anti-inflammatory drugs. *Pharmacol. Ther.* 33:383-433, 1987.

Dempsey, R.J., Roy, M.W., Meyer, K., Cowen, D.E., Tai, H.H. Development of cyclooxygenase and lipoxygenase metabolites of arachidonic acid after transient cerebral ischemia. *J.Neurosurg.* 64:118-124, 1986.

DeWitt, D.L. Prostaglandin endoperoxide synthase: regulation of enzyme expression. *Biochem. Biophys. Acta.* 1083:121-134, 1991.

DeWitt, D.L., El-Harith, E.A., Kraemer, S.A., Andrews, M.J., Yao, E.F., Armsrong, R.L., and Smith, W.L. The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. *J. Biol. Chem.* 265:5192-5198, 1990.

DeWitt, D.L., Rollins, T.E., Day, J.S., Gauger, J.A., and Smith, W.L. Orientation of the active site and antigenic determinants of prostaglandin endoperoxide synthase in the endoplasmic reticulum. *J. Biol. Chem.* 256:10375-10382, 1981.

DeWitt, D.L., and Smith, W.L. Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from the complementary DNA sequence. *Proc. Natl. Acad. Sci. U.S.A.* 85:1412-1416, 1988.

Dildy, G., van den Berg, P.P., Katz, M., Clark, S.L., Jongsma, H.W., Nijhius, J.G., and Loucks, C.A. Intrapartum fetal pulse oximetry: fetal oxygen saturation trends during labor and relation to delivery outcome. *Am. J. Obstet. Gynecol.* 171:679-684, 1994.

Fan, J.S.K., Coghlan, J.P., Denton, D.A., Scoggins, B.A., and Whitworth, J.A. Blood pressure and metabolic effects of ACTH in anephric sheep. *Clin. Exp. Pharmacol. Physiol.* 5:673-677, 1978.

Ferreira, S.H. Prostaglandins, aspirin-like drugs and analgesia. *Nature (London).* 240:200-203, 1972.

Fersht, A. Enzyme structure and function. 2nd ed., W. H. Freeman, San Francisco. 1985.

Firedman, M.A., Hirschklau, M.J., Printz, M.P., Pitlick, P.T., Kirkpatrick, S.E. Pharmacological closure of the patent ductus arteriosus in the premature infant. *N. Engl. J. Med.* 295:526-529, 1976.

Fitzpatrick, F.A., and Murphy, R.C. Cytochrome P-450 metabolism of arachidonic acid formation and biological actions of "epoxygenase"-derived eicosanids. *Pharmacol. Rew.* 40:229-241, 1988.

Gaudet, R.J., Alam, I., Levine, L. Accumulation of cyclooxygenase products of arachidonic acid metabolism in gerbil brain during reperfusion after bilateral common carotid artery occlusion. *J. Neurochem.* 35:653-658, 1980.

Gersony, W.M., Peckham, G.J., Ellison, R.C., Miettinen, O.S., Nadas, A.S. Effects of indomethacin in premature infants with patent ductus arteriosus: results of a neonatal collaborative study. *J. Pediat.* 102:895-906, 1983.

Gewirtz, H., Sasken, H., Steiner, M. and Most, A.S. Impaired arteriolar vasodilation induced by thrombosis of a coronary arterial stenosis. *Am. J. Physiol.* 249:H1154-H1166, 1985.

Goerig, M., Habenicht, A., Zeh, W., Salbach, P., Kommerell, B., Rothe, D., Nastainczyk, W., Glomset, J. Evidence for coordinate, selective regulation of eicodanoid synthesis in platelet-derived growth factor-stimulated 3T3 fibroblasts and in HL-60 cells induced to differentiate into macrophages or neutrophils. *J. Biol. Chem.* 263:19384-19391, 1989.

Goldstein, I.M., Malmsten, C.L., Kindahl, H., Kaplan, H.B., Radmark, O., Samuelsson, B. and Weissman, G. Thromboxane generation by human peripheral blood polymorphonuclear leukocytes. *J. Exp. Med.* 148:787-792, 1978.

Gubits, R.M., Burke, R.E., Casey-McIntosh, G., Bandele, A. and Munell, F. Immediate early gene induction after neonatal hypoxia-ischemia. *Mol. Brain Res.* 18:228-238, 1993.

Habib, A., Creminon, C., Frobert. Y., Grassi, J., Pradelles, P., and Maclouf, J. Demonstration of an inducible cyclooxygenase in human endothelial cells using antibodies raised against the carboxyl-terminal region of the cyclooxygenase-2. *J. Biol. Chem.* 268:23448-23454, 1993.

Hall, E., Tuan, W., and Venton, D.. Production of platelet thromboxane $A_2$ inactivates purified human platelet thromboxane synthase. *Biochem. J.* 233:637-641, 1986.

Hall, J.E., and Brands, M.W. The renin-angiotensin-aldosterone systems. In: 'The Kidney: Physiology and pathophysiology'. (Eds D. W. Seldin and G. Giebisch.) pp. 1455-1504, 1992. (Raven Press: New York.)

Hallenbeck, J.M., Furlow, T.W. Prostaglandin $I_2$ and indomethacin prevent impairment of post-ischemic brain reperfusion in the dog. *Stroke.* 10:629-637, 1979.

Hamberg, M. On the formation of thromboxane $B_2$ and 121-hydroxy-5,8,10,14-eicosatetraeroic acid (12 ho-20:4) in tissues from the guinea pig. *Biochim. Biophys. Acta.* 431:651-654, 1976.

Hamberg, M., Sevensson, J. and Samuelsson, B. Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxide. *Proc. Natl. Acad. Sci. U.S.A.* 72:2994-2998, 1975.

Hanamura, T., Shigeno, T., Asano, T., Mima, T., Takakura, K. Prostaglandin profiles in relation to local circulatory changes following focal cerebral ischemia in cats. *Stroke.* 20:803-808, 1989.

Hanigan, W.C., Kennedy, G., Roemisch, F., Anderson, R., Cusack, T., Powers, W. Administration of indomethacin for prevention of periventricular/intraventricular hemorrhage in high-risk neonates. *J. Pediatr.* 112:941-947, 1988.

Hanley, D.F., Wilson, D.A., Feldman, M.A., and Traystman, R. J. Peripheral chemoreceptor control of neurohypophyseal blood flow. *Am. J. Physiol.* 254:H742-H750, 1988.

Haurand, M., and Ullrich, V. Isolation and characterization of thromboxane synthase rom human platelets as a cytochrome p-450 enzyme. *J. Biol. Chem.* 260:15059-15067, 1985.

Hayaishi, O. Molecular mechanisms of sleep-wake regulation: roles of prostaglandins $D_2$ and $E_2$. *FASEB J.* 5:2575-2581, 1991.

Hebert, R.L., Jacobson, H.R., and Breyer, M.D. $PGE_2$ inhibits AVP-induced water flow in cortical collecting ducts by protein kinase C activation. *Am. J. Physiol.* 259:F318-F325, 1990.

Heistad, D.P., and Kontos, H.A. *Handbook of Physiology.* The Circulatory System. Peripheral Circulation and Organ Blood Flow. Bethesda, MD: American Physiological Society, 1983, p. 137-182.

Hemler, M.E., and Lands, W.E.M. Protection of cyclooxygenase activity during heme-induced destabilization. *Arch.Biochem. Biophys.* 201:586-593, 1980a.

Hemler, M.E., and Lands, W.E.M. Evidence for a peroxide-initiated free radical mechanism of prostaglandin biosynthesis. *J. Biol. Chem.* 255:6253-6261, 1980b.

Hemler, M., Lands, W.E.M., and Smith, W.L. Purification of the cyclooxygenase that forms prostaglandins: demonstration of two forms iron in the holoenzyme. *J. Biol. Chem.* 251:5575-5579, 1976.

Hernandez, M.J., Brennan, R.W., and Bowman, G.S. Autoregulation of cerebral blood flow in the newborn dog. *Brain Res.* 184:199-202, 1980.

Heymann, M.A., Rudolph, A.M., and Silverman, N.A. Closure of the ductus arteriosus in premature infants by inhibition of prostaglandin synthesis. *N. Engl. J. Med.* 295:530-533, 1976.

Hla, T., Neilson, K. Human cyclooxygenase 2 cDNA. *Proc. Natl. Acad. Sci. U.S.A.* 89:7384-7388, 1992.

Hoff, T., DeWitt, D., Kaever, V., Resch, K., and Goppelt-Struebe, M. Differentiation associated expression of prostaglandin G/H synthase in monocytic cells. *FEBS lett.* 320:38-42, 1993.

Hoffman, P.K., Share, L., Crofton, J.T., and Shade, R.E. The effect of intracerebroventricular indomethacin on osmotically stimulated vasopressin release. *Neuroendocrinology.* 34:132-139, 1982.

Holtz, M.L., Kindy, M.S., Craddock, S., Moore, R.W. and Pettigrew, L.C. Induction of PGH synthase and c-fos mRNA during early reperfusion of ischemic rat brain. *Mol. Brain Res.* 35:339-343, 1996.

Husted, D. and Wood, C.E. Immunohistochemical localization of thromboxane synthase in the ovine fetal and adult central nervous system. *J. Soc. Gynecol. Investig.* 5, 1998.(Abstract)

Ikeda, J., Nakajima, T., Gerfen, C. and Nowak, T.S. *In situ* hybridization analysis of c-fos and prodynorphin mRNA levels and distribution in gerbil hippocampus after transient ischemia. *Stroke suppl.* 21:1-16, 1990.

Inoue, M., Crofton, J.T., and Share, L. Interactions between brain acetylcholine and prostaglandins in control of vasopressin release. *Am. J. Physiol.* 261:R420-R426, 1991.

Inoue, M., Crofton, J.T., and Share, L. Interactions between the brain renin-angiotensin system and brain prostanoids in the control of vasopressin secretion. *Exp. Brain Res.* 83:131-136, 1990.

Isakson, P.C., Raz, A., Hsueh, W. and Needleman, P. Lipases and prostaglandin biosynthesis. Adv. In *Prostaglandin and Thromboxane Research,* Vol. 3, pp. 113-120. Raven Press, New York. 1977.

Itskovitz, J. and Rudolph, A.M. Denervation of arterial chemoreceptors and baroreceptors in fetal lambs in utero. *Am. J. Physiol.* 242:H916-920, 1982.

Iwamoto, H.S., and Rudolph, A.M. Role of renin-angiotensin system in response to hemorrhage in fetal sheep. *Am. J. Physiol.* 240:H848-H854, 1981.

Jacob, H.J., Alper, R.H., Grosskreutz, C.L., Lewis, S.J., and Brody, M.J. Vascular tone influences arterial pressure lability after sinoaortic denervation. *Am. J. Physiol.* 260:R359-R367, 1991.

Jones, D.A., and Fitzpatrick, F.A. Thromboxane $A_2$ synthase: modification during "suicide" inactivation. *J. Biol. Chem.* 266:23510-23514, 1991.

Jones, D.A., and Fitzpatrick, F.A. "Suicide" inactivation of thromboxane synthase $A_2$ synthase. *J. Biol. Chem.* 265:20166-20171, 1990.

Jones, S.A., Adamson, S.L., Engelberts, D., Bishai, I., Norton, J., and Cocean, F. $PGE_2$ in the perinatal brain: local synthesis and transfer across the blood brain barrier. *J. Lipid Mediat.* 6:487-492, 1993.

Jugdutt, B.I., Hutchins, G.M., Bulkley, B.H., Pitt, B., Becker, L.V. Effect of indomethacin on collateral blood flow and infarct size in the conscious dog. *Circulation.* 59:734-743, 1979.

Katsuki, H., and Okuda, S. Arachidonic acid as a neurotoxic and neurotrophic substance. Prog. *Neurobiol.* 46:607-636, 1995.

Kent, R.S., Diedrich, S.L., and Whorton, A.R.. Regulation of vascular prostaglandin synthesis by metabolites of arachidonic acid in perfused rabbit aorta. *J. Clin. Invest.* 72:455-465, 1983.

Kindy, M.S., Carney, J.P., Dempsey, R.J., and Carney, J.M. Ischemic induction of protooncogene expression in gerbil brain. *J. Mol. Neurosci.* 2:217-228, 1991.

Koistinaho, J. and Hokfelt, T. Altered gene expression in brain ischemia. *NeuroReport.* 8:i-Viii, 1997.

Koos, B.J., Mason, B.A., and Ervin, M.G. Adenosine mediates hypoxic release of arginine vasopressin in fetal sheep. *Am. J. Physiol.* 266:R215-R220, 1994.

Kujubu, D.A., B.S. Fletcher, B.C. Varnum, R.W. Lim, and H.R. Herschman. TIS10, a phorbol ester tumor promoterinducible mRNA from Swiss 3T3 cells, encodes a novel prostaglanding synthase/cyclooxygenase homologue. *J. Biol. Chem.* 226:12866-12872, 1991.

Kujubu, D. A., and Herschman, H. R. Dexamethason inhibits mitogen induction of the TIS 10 prostaglandin synthase / cyclooxygenase gene. *J. Biol. Chem.* 267:7991-7994, 1992.

Kulmacz, R.J. Topography of prostaglandin H synthase. Antiinflammatory agents and the protease-sensitive arginine 253 region. *J. Biol. Chem.* 264:14136-14144, 1989.

Kulmacz, R.J. and Lands, W.E. Stoichiometry and kinetics of the interaction of prostaglandin H synthase with anti-inflammatory agents. *J. Biol. Chem.* 260:12572-12578, 1985.

Kulmacz, R., Lands, W. Protection of prostaglandin H synthase from trypsin upon binding of heme. *Biochem. Biophys. Res. Commun.* 104:758-764, 1982.

Kusaka, M., Ohshima. T., Yokota, K., Yamamoto, S., Kumegawa, M. Possible induction of fatty acid cyclooxygenase in mouse osteoblastic cells by cAMP. *Biochim. Biophys. Acta.* 972:339-346, 1988.

Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:681-685, 1970.

Lakhdir, F.R, Tong, H., and Wood, C.E. Interaction of baroreceptors and prostanoids in the control of fetal renal blood flow. (in review).

Lands, W., and Samuelsson, B. Phospholipid precursors of prostaglandins. *Biochim. Biophys. Acta.* 164:426-429, 1968.

Lapetina, E.G., and Cuatrecasas, P. Rapid inactivation of cyclooxygenase after stimulation of intact platelets. *Proc. Nat. Acad. Sci. USA.* 76:121-125, 1979.

Larkin, S.W. and Williams, T.J. Evidence for sensory nerve involvement in cutaneous reactive hyperemia in humans. *Circ. Res.* 73:147-154, 1993.

Lee, S. H., Soyoola, E., Chanmugam, P., Hart, S., Sun, W., Zhang, H., Liou, S., Simmons, D., and Hwang, D. Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. *J. Biol. Chem.* 267:25934-25938, 1992.

Leffler, C.W., Armstead, W.A., and Shibata, M. Role of the eicosanoids in cerebral hemodynamics. In: *The Regulation of Cerebral Blood Flow*, ed. by J.W. Phyllis, pp 297-313, CRC Press, Boca Raton, 1993.

Leffler, C.W., and Busija, D.W. Prostanoids and pial arteriolar diameter in hypotensive newborn pigs. *Am. J. Physiol.* 252:H687-H691, 1987a.

Leffler, C.W., and Busija, D.W. Arachidonic acid metabolites and perinatal cerebral hemodynamics. *Seminars in Perinatalogy*. 11:31-42, 1987b.

Leffler, C.W., Busija, D.W., Armstead, W.M., Mirro, R., Beasley, D.G. Ischemia alters cerebral vascular responses to hypercapnia and acetylcholine in piglets. *Pediatr. Res.* 25:180-183, 1989.

Leffler, C.W., Busija, D.W., Beasley, D.G., and Fletcher, A.M. Maintenance of cerebral circulation during hemorrhagic hypotension in newborn pigs: Role of Prostanoids. *Circ. Res.* 59:562-567, 1986.

Leffler, C.W., Busija, D.W., Fletcher, A.M., Beasley, D.G, Hessler, J.R., Gree, R.S. Effects of indomethacin upon cerebral hemodynamics of newborn pigs. *Pediatrt. Res.* 19:1160-1166, 1985.

Loewy, A.D., and Burton, H. Nuclei of the solitary tract: efferent projections to the lower brain stem and spinal cord of the cat. *J. Comp. Neurol.* 181:421-450, 1978.

Marnett, L.J., and Maddipati, K.R. Detection of a higher oxidation state of manganese prostaglandin endoperoxide synthase. J. Biol. Chem. 267:13863-13869, 1991.

McKean, M.L., Smith, J.B., and Silver, M.J. Formation of lysophosphatidylcholine by human platelets in response to thrombin . Support for the phospholipase $A_2$ pathway for the liberation of arachidonic acid. *J. Biol. Chem.* 256:1522-1524, 1981.

Ment, L.R., Duncan, C.C., Ehrenkranz, R.A., Kleinman, C.S., Pitt, B.R., Taylor, K.J.W., Scott, D.T., Stewart, W.B., Gettner, P. Randomized indomethacin trial for prevention of intraventricular hemorrhage in very low birth weight infant. *J. Pediatr.* 107:937-943, 1985b.

Ment, L.R., Oh, W., Ehrenkranz, R.A., Philip, A.G., Vohr, B.R., Allen, W., Duncan, C.C., Scott, D.T., Taylor, K.J.W., Katz, K.H., Schneider, K.C., and Makuch, R.W. Low-dose indomethacin and prevention of intraventricular hemorrhage: a multicenter randomized trial. *Pediatrics*. 93:543-550, 1994.

Ment, L.R., Stewart, W.B., Duncan, C.C., Pitt, B.R., Rescigno, A., and Cole, J. Beagle puppy model of perinatal cerebral infarction: Acute changes in cerebral blood flow and metabolism during hemorrhagic hypotension. *J. Neurosurg.* 63:441-447, 1985a.

Merlie, J.P., Fagan, D., Mudd, J. and Needleman, P. Isolation and characterization of the complementary DNA for sheep seminal vesicle prostaglandin endoperoxide synthase (cyclooxygenase). *J. Biol. Chem.* 263:3550-3553, 1988.

Miettinen, S., Fusco, F.R., Yrjanheikki, J., Keinanen, R., Hirvonen, T., Roivainen, R., Narhi, M., Hokfelt, T. and Koistinaho, J. Spreading depression and focal brain ischemia induce cyclooxygenase-2 in cortical neurons through *N*-methyl-D-aspartic acid-receptors and phospholipase $A_2$. *Proc. Natl. Acad. Sci. USA*. 94:6500-6505, 1997.

Miyamoto, T., Ogino, N., Yamamoto, S., and Hayaishi, O. Purification of prostaglandin endoperoxide synthase from bovine vesicular gland microsomes. *J. Biol. Chem.* 251:2629-2636, 1976.

Mizuno, K., Yamamoto, S., and Lands, W.E. Effects of non-steroidal anti-inflammatory drugs on fatty acid cyclooxygenase and prostaglandin hydroperoxidase activities. *Prostaglandins*. 23:743-757, 1982.

Moncada, S., and Vane, J.R. Pharmacology and endogenous role of prostaglandin endoperoxides, thromboxane $A_2$, and prostacyclin. *Pharmacol. Rev.* 30:293-331, 1978.

Moskowitz, M.A. and Coughlin, S.R. Basic properties of the prostaglandins: current concepts of cerebrovascular disease-stroke. *Stroke*. 12:882-886, 1981.

Murota, S., Kawamura, M. and Morita, I. Transformation of arachidonic acid into thromboxane $B_2$ by the homogenates of activated macrophages. *Biochim. Biophys. Acta.* 528:507-511, 1978.

Murphy, S., and Pearce, B. Eicosanoids in the CNS: sources and effects. *Prostaglandins Leukotriences Essential Fatty Acids.* 31:165-170, 1988.

Nakao, A., Allen, M.L., Sonnenburg, W.K., and Smith, W.L. Regulation of cAMP metabolism by prostaglandin $E_2$ in the cortical and medullary thick ascending limb of Henle's loop. *Am. J. Physiol.* 256:C6520C657, 1989.

Nedergaard, M., and Diemer, N.H. Focal ischemia of the rat brain, with special reference to the influence of plasma gucose concentration. *Acta Neuropathol.* 73:131-137, 1987.

Norton, J.L., Adamson, S.L, Bocking, A.D. and Han, V.K.M. Prostaglandin-H synthase-1 (PGHS-1) gene is expressed in specific neurons of the brain of the late gestation ovine fetus. *Dev. Brain Res.* 95:79-96, 1996.

O'Banion, M.K., Miller, J.C., Chang, J.W., Kaplan, M.D., and Coleman, P.D. Interleukin-1 beta induces prostaglandin G/H synthase-2 (cyclooxygenase-2) in primary murine astrocyte cultures. *J. Neurochem.* 66:2532-2540, 1996.

Ogino, N., Ohki, S., Yamamoto, S., and Hayaishi, O. Prostaglandin endoperoxide synthase from bovine vesicular gland microsomes. *J. Biol. Chem.* 253:5061-5068, 1978.

O'Neill, G. P., and Ford-Hutchinson, A. W. Expression of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in human tissues. *FEBS Lett.* 330:156-160, 1993.

Onodera, H., Kogure, K., Ono, Y., Igarashi, K., Kiyota, Y. and Nagaoka, A. Proto-oncogene c-fos is transiently induced in the rat cerebral cortex after forebrain ischemia. *Neurosci. Lett.* 98:101-104, 1989.

Pace-Asciak, C.R., and Rangaraj, G. Prostaglandin biosynthesis and catabolism in the developing fetal sheep brain. *J. Biol. Chem.* 251:3381-3385, 1976.

Papile, L.A., Rudolph, A.M., and Heymann, M.A. Autoregulation of cerebral blood flow in the preterm fetal lamb. *Pediatrt. Res.* 19:159-161, 1985.

Pash, J.M., and Bailey, J.M. Inhibition by corticosteroids of epidemal growth factor-induced recovery of cyclooxygenase after aspirin inactivation. *FASEB J.* 2:2613-2618, 1988.

Patel, N., Cudd, T.A., Purinton, S., and Wood, C.E. Protective hemodynamic and hormonal actions of hypertonic saline are prevented by prostaglandin synthase inhibition. *FASEB J.* 11:2821, 1997.(Abstract)

Peri, K. G., Hardy, P., Li, D. Y., Varma, D. R., and Chemtob, S. Prostaglandin G/H synthase-2 is a major contributor of brain prostaglandins in the newborn. *J. Biol. Chem.* 270:24615-24620, 1995.

Pickard, J.D., MacDonell, L.A., Mackenzie, E.T., and Harper, A.M. Prostaglandin-induced effects in the primate cerebral circulation. *European Journal of Pharmacology.* 43:343-351, 1977.

Pirkle, J.C.J., and Gann, D.S. Restitution of blood volume after hemorrhage: mathematical description. *Am. J. Physiol.* 228:821-827, 1975.

Pourcyrous, M., Leffler, C.W., Bada, H.S., Korones, S.B., and Busija, D.W. Cerebral blood flow responses to indomethacin in awake newborn pigs. *Pediatr. Res.* 35:565-570, 1994.

Pourcyrous, M., Leffler, C.W., and Busija, D.W. Role of prostaglandin cerebrovascular responses to asphyxia and reventilation in newborn pigs. *Am. J. Physiol.* 259:662-667, 1990.

Purves, M.J., and James, I.M. Observations on the control of cerebral blood flow in the sheep fetus and newborn lamb. *Circ. Res.* 25:651-667, 1969.

Raff, H., Kane, C., and Wood, C.E. Vasopressin responses to hypoxia and hypercapnia in late-gestation fetal sheep. *Am. J. Physiol.* 260:R1077-R1081, 1991.

Raff, H., Shinsako, J., and Dallman, M.F. Renin and ACTH responses to hypercapnia and hypoxia after chronic carotid chemodenervation. *Am. J. Physiol.* 247:R412-R417, 1984.

Rando, R. Mechanism-based enzyme inactivators. *Pharmacol. Rev.* 36:111-142, 1984.

Raz, A. and Needleman, P. Differential modification of cyclooxygenase and peroxidase activities of prostaglandin endoperoxide synthase by proteolytic digestion and hydroperoxides. *Biochem. J.* 269:603-607, 1990.

Raz, A., Wyche, A., and Needleman, P. Temporal and pharmacological division of fibroblast cyclooxygenase expression into transcriptional and translational phases. *Proc. Natl. Acad. Sci. U.S.A.* 86:1657-1661, 1989.

Rittenhouse-Simmons, S., Russell, F.A. and Deykin, D. Mobilization of arachidonic acid in human platelets: kinetics and $Ca^{2+}$ dependency. *Biochim. Biophys. Acta.* 488:370-380, 1976.

Robillard, J.E., Gomez, R.A., Meernik, J.G., Kuehl, W.D., and VanOrden, D. Role of angiotensin II on the adrenal and vascular responses to hemorrhage during development in fetal lambs. *Circ. Res.* 50:645-650, 1982.

Robillard, J.E., Weitzman, R.E., Fisher, D.A., and Smith, F.G. The dynamics of vasopressin release and blood volume regulation during fetal hemorrhage in the lamb fetus. *Pediatr. Res.* 13:606-610, 1979.

Rollins, T.E., and Smith, W.L. Subcellular localization of prostaglandin-forming cyclooxygenase in Swiss mouse 3T3 fibroblasts by electron microscopic immunocytochemistry. *J. Biol. Chem.* 255:4872-4875, 1980.

Rome, L.H. and Lands, W.E. Structural requirements for time-dependent inhibition of prostaglandin biosynthesis by anti-inflammatory drugs. *Proc. Natl. Acad. Sci. U.S.A.* 72:4863-4865, 1975.

Rose, J.C., Meis, P., and Morris, M. Ontogeny of endocrine (ACTH, vasopressin, cortisol) responses to hypotension in lamb fetuses. *Am. J. Physiol.* 240:E656-E661, 1981.

Rosen, G.D., Birkenmeier, T.M., Raz, A., and Holtzman, M.J. Identification of a cyclooxygenase-related gene and its potential role in prostaglandin formation. *Biochem. Biophys. Res. Commun.* 164:1358-1365, 1989.

Roth, G.J., and Siok, C.J. Acetylation of the NH2-termianl serine of prostaglandin synthetase by aspirin. *J. Biol. Chem.* 253:3782-3784, 1978.

Roth, G.J., Siok, C.J. and Ozols, J. Structural characteristics of prostaglandin synthase from sheep vesicular gland. *J. Biol. Chem.* 255:1301-1304, 1980.

Roth, G.J., Stanford, N., and Majerus, P.W. Acetylation of prostaglandin synthase by aspirin. *Proc. Natl. Acad. Sci. U.S.A.* 72:3073-3076, 1975.

Ruppert, C. and Wille, W. Proto-oncogene c-fos is highly inducible by disruption of neonatal but not mature brain tissue. *Mol. Brain Res.* 2:51-56, 1987.

Samuelsson, B., Goldyne, M., Granstrom, E., Hamberg, M., Hammarstrom, S., and Malmsten, C. Prostaglandins and thromboxanes. *Annu. Rev. Biochem.* 47:997-1029, 1978.

Sawchenko, P.E., and Swanson, L.W. The organization of noradrenergic pathways from the brain stem to the paraventricular and supraoptic nuclei in the rat. *Brain Res.* 4:275-325, 1982.

Schwartz, J., and Reid, I.A. Effect of vasopressin blockade on blood pressure regulation during hemorrhage in conscious dogs. *Endocrinology.* 109:1778-1780, 1981.

Share, L. Effects of carotid occlusion and feft atrial distention on plasma vasopressin titer. *Am. J. Physiol.* 208:219-223, 1965.

Shen, R.F., Tai, T.T. Monoclonal antibodies to thromboxane synthase from porcine lung. Production and application to development of a tandem immunoradiometric assay. *J. Biol. Chem.* 261:11585-11591, 1986.

Shimazu, M , Mizushima, H., Sasaki, K., Arai, Y., Matsumoto, K., Shioda, S. and Nakai, Y. Expression of c-fos in the rat cerebral cortex after focal ischemia and reperfusion. *Brain Res. Bull.* 33:689-697, 1994.

Shimizu, T., and Wolfe, S.L. Arachidonic acid casade and signal transduction. *J. Neuchem.* 55:1-12, 1990.

Shohami, E., Rosenthal, J., Lavy, S. The effect of incomplete cerebral ischemia on prostaglandin levels in rat brain. *Stroke.* 13:494-499, 1982.

Simmons, D.L., W. Xie, J.G. Chipman, and G.E. Evett. Multiple cyclooxyzgenases: cloning of a mitogen-inducuble form. In: *Prostaglandings, Leukotrienes, Lipoxiins, and PAF*, edited by J.M. Baily. New York: Plenum, p. 67-78, 1991.

Siren, A.L. Central cardiovascular and thermal effects of prostaglandin $E_2$ in rats. *Acta Physiol Scand.* 116:229-234, 1982.

Smith, E.F., Lefer, A.M. and Smith, J.B. Influence of thromboxane inhibition on the severity of myocardial ischemia in cats. *Can. J. Physiol.* 58:294-300, 1980.

Smith, W.L. Localization of enzymes responsible for prostaglandin formation. In: Willis AL. ed. *Handbook of Eicosanoids: Prostaglandins and Related Lipids.* CRC Press, Boca Raton. pp:175-184, 1987.

Smith, W.L. The eicosanoids and their biochemical mechanisms of action. *Biochem. J.* 259:315-324, 1989.

Smith, W.L. Prostanoid biosynthesis and mechanisms of action. *Am. J. Physiol.* 263:F181-F191, 1992.

Smith, W., and Lands, W. Oxygenation of unsaturated fatty acids by soybean lipoxygenase. *J. Biol. Chem.* 247:1038-1044, 1971.

Smith, W.L., and Lands, W.E.M. Oxygenation of polyunsaturated fatty acids during prostaglandin biosynthesis by sheep vesicular glands. *Biochemistry.* 11:3276-3285, 1972.

Smith, W.L., and Marnett, L.J. Prostaglandin endoperoxide synthase: structure and catalysis. *Biochim. Biophys. Acta.* 1083:1-17, 1991.

Smith, W.L., Marnett, L.J., DeWitt, D.L. Prostaglandin and thromboxane biosynthesis. *Pharmacol* Ther. 49:153-179, 1991.

Sonnenburg, W.K., and Smith, W.L. Regulation of cAMP metabolism in rebbit cortical collecting tubule cells by prostaglandins. *J. Biol. Chem.* 263:6155-6160, 1988.

Sonnenburg, W.K., Zhu, J., and Smith, W.L. A prostaglandin E receptor coupled to a pertusis toxin sensitive guanine nucleotide regulatory protein in rabbit cortical collecting tubule cells. *J. Biol. Chem.* 265:8479-8483, 1990.

Spyer, K. M. Baroreceptor sensitive neurons in the anterior hypothalamus of the cat. *J. Physiol.* 224:245-257, 1972.

Stevens, M.K., Yaksh, T.L. Time course of release in vivo of $PGE_2$, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and $TxB_2$ into the brain extracellular space after 15 min of complete global ischemia in the presence and absence of cyclooxygenase inhibition. *J. Cereb. Blood Flow Metab.* 8:790-798, 1988.

Tai, T.C., MacLusky, N.J. and Adamson, S.L. Ontogenesis of prostaglandin $E_2$ binding sites in the brainstem of the sheep. *Brain Res.* 652:28-30, 1994.

Thames, M.D., Jarecki, M., and Donald, D.E. Neural control of renin secretion in anesthetized dogs. Interaction of cardiopulmonary and carotid baroreceptors. *Circ. Res.* 42:237-245, 1978.

Thompson, M.E. and Hege, G.A. Inhibition of corticotropin secretion by hypothalamic administration of indomethacin. *Neuroendocrinology.* 25:212-220, 1978.

Toh, H., Ichikawa, A., and Narumiya, S. Molecular evolution of receptors for eicosanoids. *FEBS. Lett.* 361:17-21, 1995.

Tong, H., Lakhdir, F., Wood, C.E. Endogenous prostanoids medulate the ACTH and AVP responses to hypotension in late-gestation fetal sheep. *Am. J. Physiol.* 275:R735-R741, 1998.

Tong, H and Wood, C.E. Expression of prostaglandin endoperoxide synthase-2 in response to cerebral hypoperfusion in late-gestation fetal sheep brain. *Proc. 45th Annual Meeting of the Soc. Gynecol. Invest.* Abstract #108. 1998.

Trachte, G.J. Thromboxane agonist (U46619) potentiates norepinephrine efflux from adrenergic nerves. *J. Pharmacol. Exp. Ther.* 237:473-477, 1986.

Traystman, R.J. Control of cerebral blood flow. In: Vanhoutte, P.M., Leusen, I. ed. *Vasodilation.* Raven Press, New York, pp39-44, 1981.

Tweed, W.A., Cote, J., Pash, M., and Lou, H. Arterial oxygenation determines autoregulation of cerebral blood flow in the fetal lamb. *Pediat. Res.* 17:246-249, 1983.

Van der Ouderaa, F.J., Buytenhek, M., Nugteren, D.H. and van Dorp. D.A. Purification and characterization of prostaglandin synthase from sheep vesicular glands. *Biochim. Biophys. Acta.* 487:315-331, 1977.

Walsh, C.I. Suicide substrates: mechanism-based inactivators. *Annu. Rev. Biochem.* 53:493-535, 1984.

Wang, L.H., Hajibeigi, A., Xu, X.M., Loose-Mitchell, D., W, K. Characterization of the promoter of human prostaglandin H synthase-1 gene. *Biochem. Biophys. Res. Commun.* 190:406-411, 1993.

Weidmann, M.J., Peskar, B.A., Wrogemann, K., Rietschel, E.T., Staudinger, H.J. and Fisher, H. Prostaglandin and thromboxane synthesis in a pure macrophage population and

the inhibition by E-type prostaglandins of chemiluminescence. *FEBS Lett.* 89:136-140, 1978.

White, R.P., and Hagen, A.A. Cerebrovascular actions of prostaglandins. *Pharmacol. Ther.* 18:313-331, 1982.

Whittake, N., Bunting, S., Salmon, J., Moncada, S., Vane, J., Johnson, R.A., Morton, D., Kinner, J., Gorman, R., McGurie, J., and Sun, F. The chemical structure of prostaglandin. *Prostaglandins.* 12:915-928, 1976.

Wimsatt, J., Nathanielsz, P.W., Sirois, J. Induction of prostaglandin endoperoxide synthase isoform-2 in ovine cotyledonary tissues during late gestation. *Endocrinology.* 13:1068-1073, 1993.

Winer, B.J. *Statistical Principles in Experimental Design.* New York: McGraw-Hill, p. 514-603, 1971.

Wolfe, L.S., Coceanim, F. The role of prostaglandins in the central nervous system. *Annul Rev Physiol.* 41:669-684, 1979.

Wolfe, L.S., Rostworoski, K., and Marion, J. Endogenous formation of the prostaglandin endoperoxide metabolite, thromboxane $B_2$, by brain tissue. *Biochem. Biophys. Res. Commun.* 70:907-913, 1976.

Wong, W. Y., and Richards, J. S. Induction of prostaglandin H synthase in rat preovulatory follicles by gonadotropin-releasing hormone. *Endocrinology.* 130:3512-3521, 1992.

Wood, C.E. Sinoaortic denervation attenuates the reflex responses to hypotension in fetal sheep. *Am. J. Physiol.* 256:R1103-R1110, 1989.

Wood, C.E. Baroreflex and chemoreflex control of fetal hormone secretion. *Reprod. Fertil. Dev.* 7:479-489, 1995a.

Wood, C.E. Fetal responses to carotid occlusion: immaturity of buffering systems. *Am. J. Physiol.* 268:R343-R348, 1995b.

Wood, C.E. and Chen, H.G. Acidemia stimulates ACTH, vasopressin, and heart rate responses in fetal sheep. *Am. J. Physiol.* 257:R344-R349, 1989.

Wood, C.E., Chen, H.G., and Bell, M.E. Role of vagosympathetic fibers in the control of adrenocorticotropic hormone, vasopressin, and renin responses to hemorrhage in fetal sheep. *Circ.Res.* 64: 515-523, 1989.

Wood, C.E., Cudd, T.A., Kane, C., and Engelke, K. Fetal ACTH and blood pressure responses to thromboxane mimetic U46619. *Am. J. Physiol.* 265:R858-R862, 1993.

Wood, C.E., Kane, C., and Raff, H. Peripheral chemoreceptor control of fetal renin responses to hypoxia and hypercapnia. *Circ. Res.* 67:722-732, 1990.

Wood, C.E., Keil, L.C., and Rudolph, A.M. Hormonal and hemodynamic responses to vena caval obstruction in fetal sheep. *Am. J. Physiol.* 243:E278-E286, 1982.

Wood, C.E., Keil, L.C., and Rudolph, A.M. Carotid arterial control of vasopressin secretion in sheep. *Am. J. Physiol.* 247:R589-R594, 1984.

Wood, C.E., Purinton, S., Cudd T.A. Immunoreactive thromboxane synthase is measurable in ovine fetal hypothalamus as early as 86 days' gestation. *Prostaglandins.* 54:569-579, 1997.

Wood, C.E. and Rudolph, A.M. Carotid vascular control of ACTH secretion in lambs. *Am. J. Physiol.* 244:E555-E559, 1983.

Wu, K.K., Hatzakis, H., Lo, S.S., Seong, D.C., Sanduja, S.K., and Tai, H.H. Stimulation of de novo synthesis of prostaglandin G/H synthase in human endothelial cell by phorbol ester. *J. Biol. Chem.* 263:19042-19047, 1988.

Xie, W.L., Chipman, J.G., Robertson, D.L., Erikson, R.L., and Simmons, D.L. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc. Natl. Acad. Sci. U.S.A.* 88:2692-2696, 1991.

Xie, W.L., Merrill, J.R., Bradshaw, W.S., and Simmons, D.L. Structural determination and promotor analysis of the chicken mitogen-inducible prostaglandin G/H synthase gene and genetic mapping of the murine homolog. *Arch. Biochem. Biophys.* 300:247-252, 1993.

Yamagata, K., Andreasson, K. I., Kaufmann, W. E., Barnes, C. A., and Worley, P. F. Expression of a mitogen-inducible cyclooxygenase in brain neurons: Regulation by synaptic activity and glucocorticoids. *Neuron.* 11:371-386, 1993.

Yamamoto, M., Share, L., and Shade, R.E. Vasopressin release during ventriculo-cisternal perfusion with prostaglandin E2 in the dog. *J. Endocr.* 71:325-331, 1976.

Yokota, K., Kusaka, M., Ohshima. T., Yamamoto, S., Kurihara. N., Yoshino, T., and Kumegawa, M. Stimulation of prostaglandin $E_2$ synthesis in cloned osteoblastic cells of mouse by epidemal growth factor. *J. Biol. Chem.* 261:15410-15415, 1986.

Yokoyama, C., Takai, T., and Tanabe, R. Primary structure of sheep prostaglandin endoperoxide synthase deduced from cDNA sequence. *FASEB Lett.* 231:347-351, 1988.

Yokoyama, C., and Tanabe, R. Cloning of human gene encoding prostaglandin endoperoxide synthase and primary structure of the enzyme. *Biochem. Biophys. Res. Commun.* 165:888-894, 1989.

Yoshimura, T., Magness, R.R., and Rosenfeld, C.R. Angiotensin II and alpha-agonist II. Effects on ovine fetoplacental prostaglandins. *Am. J. Physiol.* 251:H473-H479, 1990.

Young, I.R. and Thorburn, G.D. Prostaglandin $E_2$, fetal maturation and ovine partuition. *Aust. NZ. J. Obstet. Gynaecol.* 34:3:342-346, 1994.

Zenser, T.V., Herman, C.A., Gorman, R.R. and Davis, B.B. Metabolism and action of the prostaglandin endoperoxide $PGH_2$ in rat kidney. *Biochem. Biophys. Res. Commun.* 79:357-363, 1977.

Zhang, V., O'Sullivan, M., Hussain, H., Roswit, W.T. and Holtzman, M.J. Molecular cloning, functional expression, and selective regulation of ovine prostaglandin H synthase-2. *Biochem. Biophys. Res. Commu.* 227:499-506, 1996.

# BIOGRAPHICAL SKETCH

Haiyan Tong was born on January 30, 1966, in Jinzhou city, Liaoning Province, the People's Republic of China. After graduation from Jinzhou High School, she entered China Medical University with a dream to become a medical doctor. She spent five years in medical school and graduated with a medical degree (M.D.) in Clinical Medical Sciences in 1988. Then she did her residency in the Department of Obstetrics and Gynecology, Beijing Friendship Hospital. She entered the Ph.D. program at the University of Florida in August 1994 with a major in physiology. She was awarded a International Student Academic Award from the University of Florida in 1995. She was also awarded a Graduate Student Fellowship from the American Heart Association, Florida Affiliate, from 1996 to 1998. She expects to graduate in December 1998. In November 1998, she was employed as a research fellow at the National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park in North Carolina.

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